

AN INVESTIGATION INTO THE ROLE OF VARIOUS FOOD COMPONENTS ON THE SOLUBILITY AND DISSOLUTION OF PROPYLTHIOURACIL AND PENICILLIN V IN PAEDIATRICS

AN INVESTIGATION INTO THE ROLE OF OESTROGEN RECEPTOR β IN MEDULLARY THYROID CANCER

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the degree of MASTERS IN RESEARCH

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Project 1:

**AN INVESTIGATION INTO THE ROLE
OF VARIOUS FOOD COMPONENTS ON
THE SOLUBILITY AND DISSOLUTION
OF PROPYLTHIOURACIL AND
PENICILLIN V IN PAEDIATRICS**

This project is submitted in partial fulfilment of
the requirements for the award of the MRes

Abstract

Oral drug absorption, and therefore efficacy, can be affected by food-drug interactions. Consequently, during drug development, clinical studies are carried out in adults to determine the effect of food on the pharmacokinetic profile of the drug. However, such studies are rarely carried out in children, despite sometimes having different food-drug effects to adults. Furthermore, as medicines are often administered to children with food, it is important to understand which foods can and cannot be used in the administration of different drugs. The aim was to see which food components have the greatest effect on the absorption of two drugs commonly used in paediatrics, in order to discover which foods can be used to administer these drugs to children. This was achieved by undertaking solubility and dissolution experiments on propylthiouracil and penicillin V in media representing the fasted and fed states, and ranges of pH, fat, calcium, protein, and fibre contents. All concentrations of protein used caused a significant reduction in propylthiouracil solubility and dissolution. Furthermore, propylthiouracil dissolution was significantly reduced by fed state solutions, and media at more neutral pH, and high fat or calcium contents. Penicillin dissolution was strongly affected by media of low pH and high protein concentrations. In conclusion, propylthiouracil is best administered to children with more acidic foods, such as apple sauce or fruit juice, while more neutral foods high in fat, calcium and protein, for example milk and yoghurt, should be avoided. Conversely, penicillin V should be delivered with neutral foods, but not acidic foods.

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List of Abbreviations

AUC	– area under the absorption curve
Blank FaSSIF	– fasted state simulated intestinal fluid without bile salts and fats
Blank FeSSIF	– fed state simulated intestinal fluid without bile salts and fats
Ca ²⁺	– calcium
C _{max}	– peak plasma concentration of drug
FaSSIF	– fasted state simulated intestinal fluid containing bile salts and fats
FeSSIF	– fed state simulated intestinal fluid containing bile salts and fats
HPLC	– high performance liquid chromatography
Penicillin V	– phenoxymethylpenicillin
T _{max}	– time to reach C _{max}

1.0 Introduction

1.1 Food-drug interactions

It is widely known that food-drug interactions can affect the oral absorption, and therefore the efficacy and/or toxicity, of a drug [1-3]. As a result, during drug development, fed effect clinical studies must be undertaken to assess if, compared to the fasted state, food causes a clinically significant difference in the peak plasma concentration (C_{max}) of the drug, the time to C_{max} (T_{max}), and the area under the absorption curve (AUC), where plasma concentration of the drug is plotted against time. Food can affect the amount of drug absorbed, which has important clinical significance. If food reduces the absorption of the drug, then the patient does not receive the desired dosage, thus reducing the drug's efficacy. However, it is also possible for food to increase the absorption of a drug, resulting in the patient receiving more than the desired dosage. This can cause the patient to experience adverse effects of the drug, which is not desirable. Alternatively, food can affect the rate of drug absorption, which generally has less clinical significance, but can have greater impact when using modified-release formulations [1-4]. For example, a slow-release drug is designed to take longer to break down in the gut, but still provide the desired dosage. However, if food slows transit time through the gut, the slow-release drug is releasing its contents into the gut for a longer period of time, resulting in increased drug absorption, which may lead to increased adverse effects, as previously stated. These fed effect studies provide important information when determining the most appropriate dosage and timing of new drugs. Due to the importance of food-drug interactions, there is detailed guidance on conducting fed

effect studies in adult populations, which are strictly adhered to when carrying out such studies [5, 6].

1.2 Effects of the different food components

Not only can food interact with the drug itself to affect its solubility and absorption in a physico-chemical food-drug interaction, but food can also affect gastrointestinal physiology. However, the composition of food can have different results, because the various components of food exert different effects on the gastrointestinal system [7]. The effects of assorted food components (pH, fat, calcium, protein and fibre) are illustrated in Table 1 below.

	Effect on gastrointestinal tract	Potential effect on drug absorption
pH	The presence of food increases gastric (stomach) pH.	At increased pH, there is increased absorption of weak acid drugs, but decreases absorption of weak base drugs.
Fat	Increases secretion of bile and pancreatic juices. Slows gastric emptying.	Increases solubility of poorly water-soluble drugs, resulting in a larger concentration gradient, which increases the absorption of the drug.
Calcium	Increases chelation.	Reduces absorption of chelated drugs
Protein	Increases gastric pH. Increased binding.	Reduces absorption of bound drugs and of weak base drugs.
Fibre	Increases viscosity within the gastrointestinal tract, which reduces rate of diffusion. Delays gastric emptying. Increased binding.	Reduced absorption due to increased binding and viscosity.

Table 1 – A summary of the physiological effect of various food components on the gastrointestinal system, and of the physico-chemical effects of various food components on the absorption of drugs. Gastric emptying is the emptying of the stomach contents into the small intestine, where absorption occurs. Chelation is the binding of drugs to metal cations, such as calcium ions (Ca^{2+}). Drugs can also bind to protein and fibre molecules within the gastrointestinal tract. Both chelation and binding reduces drug absorption. (Adapted from [7])

1.2.1 Standard meal

The regulatory guidance mentioned in Section 1.1 states that a standard breakfast should be used in fed effect clinical studies. This meal is usually in the form of a fried breakfast, because this meal is high-fat, with a calorific content of 800-1000kcal, with fat constituting 50-65% of this energy, carbohydrates 25-30%, and protein 15-20%. This will have the largest effect on gastrointestinal physiology, thus providing a 'worst case scenario' in terms of the effect food can have on the oral absorption of the drug in question [5, 6].

1.3 Food-drug effects in paediatrics

Despite there being strict regulations on fed effect clinical studies in adults, very little has been done to investigate food-drug interactions in paediatrics. This is because data from adults is usually extrapolated down to paediatrics, thus assuming that children will respond to the drug in the same way as adults [8, 9]. However, as stated below, there are fundamental differences between adults and paediatrics that result in different food-drug interactions in the two populations, as described in Section 1.3.1 below. This means that children may not be receiving the maximum therapeutic benefit from a drug, or might even be receiving increased toxicity from a drug, due to altered extents and/or rates of drug absorption. In fact, fed effect studies actually carried out in paediatric populations have revealed several drugs with different responses to food compared to the data from adults. Some of these are described below. This shows that we cannot assume that food will affect a drug in the same way in adults and children, thus highlighting the importance of carrying out fed effect clinical studies in paediatric populations.

However, due to the absence of regulations on fed effects clinical studies in paediatric populations, there is no standard meal for the various age ranges. This means that, unlike adult studies, where the standard meal allows studies to be compared, there is a huge variety in the meal given in food effect studies in paediatric populations. This means that it is hard to compare the data from different studies, which creates confusion.

1.3.1 Drugs with different food-drug effects in paediatrics compared to adults

As previously stated in Section 1.3, several drugs have been found to have different food-drug effects when tested in fed effect clinical studies in paediatric populations compared to in adult populations.

Three of these drugs are amoxicillin, ampicillin and penicillin V (phenoxymethylpenicillin), which are all used to treat bacterial infections [10-12]. When tested in adults with a 500mg dose administered after the standard breakfast, there was no effect on amoxicillin absorption [13], but there was reduced C_{max} and AUC of ampicillin [13]. However, when an oral suspension of amoxicillin or ampicillin at a dose of 15 or 25mg/kg was tested in paediatrics (4-45 months, mean age of 27 months) with 4oz milk or formula, the opposite effect was seen, i.e. there was reduced C_{max} of amoxicillin at 15mg/kg dosage (although not at 25mg/kg dosage) [14], whereas there was no effect on ampicillin absorption [14]. It is recommended to take penicillin V in the fasted state in adults due to slight effects in its absorption [15]. Two different studies in paediatric populations show that both the C_{max} and AUC of penicillin V were reduced when administered with breakfast in older children (6 months – 5 years) [16] and with milk in infants [17], confirming that food, particularly milk, reduces penicillin V absorption.

Another drug where food has no effect on its absorption in adults is propylthiouracil [18], which is used to treat hyperthyroidism [19]. However, when administered with food in paediatric populations, T_{max} was increased, while C_{max} was reduced and AUC was variable [20].

Conversely, desmopressin is an example of a drug that has a known food-drug effect in adults, but not in children. Desmopressin is an analogue of vasopressin, which acts on the kidneys to reduce urine production, and is used in the treatment of conditions such as diabetes insipidus (not diabetes mellitus) and bedwetting in older children [21]. In adults, food reduces both C_{max} and AUC of desmopressin [22]. When given with the standard meal in older children (mean age 12.7 years), the absorption of desmopressin was not affected [23].

1.4 Differences between adults and paediatrics

1.4.1 Physiology

	Neonate (0-27 days)	Infant (1-23 months)	Child (2-11 years)	Adolescent (12-18 years)	Adult (18+ years)
Stomach pH	6-8*	1.4	1.5	1.5	1-2.5
Intestinal pH	–	–	6.4-7.4	6.4-7.4	6-7.5
Transit time through stomach	54-82mins	12-70mins	12-70mins	12-138mins	5-120mins
Transit time through small intestine	4hrs	4hrs	3-7.5hrs	3-7.5hrs	3-4hrs

* Neonates are born with a stomach pH of 6-8. A few hours after birth (or longer in premature neonates), the pH lowers to 1.5-3. Between 1-10 days after birth, stomach pH rises back to pH 6-7, staying at this pH for 20-30 days. After this, stomach pH gradually falls to pH 1-2 over a period of 20-30 months.

Table 2 – Summary of the pH values of the stomach and intestine, and of the transit time through the stomach and intestine, in a variety of age ranges. Due to the presence of amniotic fluid in the gastrointestinal tract, neonates are born with a stomach pH of 6-8, which lowers a few hours after birth, and rises again to pH 6-7 due to reduced acid secretion in the stomach. As this secretion increases with age, stomach pH falls gradually to pH 1-2 after approximately two years. There is little data on the intestinal pH of neonates and infants, but there is little difference between intestinal pH in children, adolescents and adults. Transit time through the stomach (or gastric emptying time – emptying of the stomach’s contents into the small intestine) becomes more variable with age, due to the effects of a more varied diet on the gastrointestinal system. Transit time through the small intestine is longer in neonates and infants, as it gradually falls with age. (Adapted from [4])

Table 2 illustrates some of the differences in the gastrointestinal physiology of adults and children. These have certain implications for drug absorption, as detailed below.

Neonates have a variable gastric (stomach) pH, which may have an impact on oral drug absorption, particularly on the absorption of weak acids and weak bases (Table 1). The reduced gastric secretion in neonates results in fewer active gastric enzymes, which also impacts on drug absorption [4]. However, this increased gastric pH can mean greater absorption of acid labile drugs, such as penicillin [4], as the drug is more stable at higher pH.

As the main site of drug absorption is the small intestine, the rate of emptying the stomach contents into the small intestine (known as gastric emptying) is often the rate limiting step in drug absorption. Up until six months of age, infants have slower gastric emptying, resulting in slower drug absorption and subsequently slower T_{max} [4].

Irregular intestinal motility in neonates and infants results in variable transit times of food through the small and large intestines, ranging from 8-96 hours, compared to 2-48 hours in adults [4]. Conversely, children tend to have a faster gut transit time, which may result in incomplete absorption of modified-release drugs because the drug passes through without releasing the whole dose [4]. These differences in transit time results in different rates and extents of drug absorption, which has a knock-on effect on bioavailability and efficacy of the drug.

1.4.2 Diet

	Calorific Input	Carbohydrate	Fat	Protein
Infants (1-3 years)	900-1000kcal	45-65%	30-40%	5-20%
Children (4-13 years)*	<u>Female</u> 1200-1600kcal <u>Male</u> 1400-1800kcal	45-65%	25-35%	10-30%
Adolescents (14-18 years)	<u>Female</u> 1800kcal <u>Male</u> 2200kcal	45-65%	25-35%	10-30%
Adult (18+ years)	<u>Female</u> 2000kcal <u>Male</u> 2500kcal	45-65%	20-35%	10-35%

* Lower end of calorific input range is for the lower age range (4-8 years); higher end is for the older age range (9-13 years)

Table 3 – Recommended calorific input and proportion of carbohydrate to fat to protein in the diet for different age ranges and genders. Calorific input increases with age, with males requiring more calories than females. The ratio of carbohydrate to fat to protein is similar for most age ranges, but infants have different nutritional needs, as fat contributes a greater percentage to the diet, while less protein is required. (Adapted from [24])

As shown in Table 3, adults and children have different nutritional needs. Fat is the main energy source for infants less than six months old, but as the infant matures, this slowly changes so that carbohydrate becomes the main energy source [24]. This has implications for the physiology of the gut and for oral drug absorption, as a high fat content slows gastric emptying, but also enhances the solubility of less water-soluble drugs, as shown in Table 1.

Table 3 also illustrates that the standard meal for food-effect clinical studies in adults (see Section 1.2.1 above) is the worst case scenario of a meal, considering the daily recommended calorific intake and composition for adults. The standard meal is nearly half the daily recommended calories, and has almost double the daily

recommended fat content. As previously stated in Table 1, this means that the digestive system will be greatly altered, having the greatest effect on drug absorption.

1.4.3 Compliance

The absence of guidelines on fed effect clinical studies in paediatric populations like those for adults has resulted in most paediatric pharmacokinetic studies being undertaken in the fasted state. However, this is not representative of administering drugs to paediatric populations. Children are often unable and/or unwilling to swallow tablets with water alone. Consequently, drugs are often crushed and mixed with soft foods, such as apple sauce or yoghurt, or drinks, such as fruit juice or milk, in order to make the drug both more palatable and easier to consume, thus facilitating the administration of the drug [25, 26]. This is not just done in the home – medical professionals will also mix drugs with food when administering to paediatrics [25], and the British National Formulary for Children (BNF-C) recommend certain drugs to be mixed with food or drink in order to facilitate its administration [26].

1.5 Aims

As previously stated, food is known to affect the oral absorption of many drugs, and therefore it is often recommended to take them on an empty stomach. However, little is known about how food affects drug absorption in paediatric populations, despite this being a very important issue, as children are rarely administered drugs without food or drink.

The aim of this project is to identify which component(s) of food (pH, fat, calcium, protein or fibre) have the strongest influences on the solubility and dissolution of

propylthiouracil and penicillin V – two drugs commonly used in paediatrics that have been found to have different food-drug effects in paediatrics compared to adult populations (see Section 1.3.1).

Subsequently, this information could be used to best advise both parents and medical professionals which foods can be used, and which are best avoided, when administering these drugs to paediatric populations.

2.0 Materials and Methods

2.1 Preparation of solubility and dissolution media

2.1.1 Fasted State Simulated Intestinal Fluid (FaSSIF) and Fed State Simulated Intestinal Fluid (FeSSIF)

To make 1L blank FaSSIF or blank FeSSIF; 0.348g sodium hydroxide pellets (Sigma Aldrich), 4.470g sodium dihydrogen phosphate dehydrate (Fischer Scientific) and 6.186g sodium chloride (Fischer Scientific); or 4.04g sodium hydroxide pellets, 144ml 1M acetic acid solution (Sigma Aldrich) and 11.874g sodium chloride, respectively, were added to approximately 900ml deionised water and stirred until dissolved using a magnetic stirrer. The pH was adjusted to pH 6.8 or pH 5.0, respectively, using 0.2M NaOH (Thermo Scientific) or 0.2M HCl (Thermo Scientific) as required, then transferred to a 1L volumetric flask and made up to volume with deionised water.

To make 1L FaSSIF or FeSSIF; 1.65g sodium taurocholate (Sigma Aldrich) and 0.59g lecithin (Lipoid EPC); or 8.25g sodium taurocholate and 2.95g lecithin, respectively, were added to 200ml blank FaSSIF or blank FeSSIF, respectively, and

stirred overnight on a magnetic stirrer to form a clear micellar solution. On day 2, these solutions were transferred to separate 1L volumetric flasks and made up to volume with blank FaSSIF or blank FeSSIF, respectively.

Solutions were made fresh immediately before the first use, then stored at room temperature until stocks were used up before making up from fresh again.

2.1.2 pH buffer solutions

To make 1L solutions at pH 2.6, pH 4.6, pH 6.6 or pH 7.4; 891ml, 532.5ml, 272.5ml, or 91.5ml 0.1M citric acid (Fischer Scientific), respectively, were mixed together with 109ml, 467.5ml, 727.5ml, or 908.5ml 0.2M di-sodium hydrogen phosphate dehydrate (BDH Laboratory Supplies), respectively.

The solutions above represent a highly acidic diet, a less acidic diet, intestinal pH and blood pH, respectively.

Solutions were made fresh immediately before the first use, then stored at 4°C until stocks were used before making up from fresh again.

2.1.3 Fat

To make 1L solutions at 6%, 22% or 34% fat; 27.08g Light Coffee-Mate (Nestle), 150g First Infant Milk (Cow and Gate), or 27.08g Original Coffee-Mate (Nestle), respectively, were added (as per manufacturer's instructions) to 1L volumetric flasks and made up to volume with blank FaSSIF. The solutions were mixed via manual inversion.

Solutions were made fresh immediately before the first use, then stored at 4°C for up to one week before making up from fresh again.

2.1.4 Calcium

To make 1L solutions at 0.2mg/ml, 0.5mg/ml, 1mg/ml or 2mg/ml calcium; 200mg, 500mg, 1000mg, or 2000mg calcium chloride (Sigma Aldrich), respectively, were added to 1L volumetric flasks and made up to volume with blank FaSSIF and stirred until dissolved using a magnetic stirrer.

Solutions were made fresh immediately before the first use, then stored at 4°C until stocks were used before making up from fresh again.

2.1.5 Protein

To make 1L solutions at 2%, 4%, 6% or 8% protein; 20g, 40g, 60g, or 80g casein (Fischer Scientific) respectively was added to 1L volumetric flasks and made up to volume with blank FaSSIF. The solutions were mixed via manual inversion.

Solutions were made fresh immediately before the first use, then stored at 4°C until stocks were used before making up from fresh again.

2.1.6 Fibre

To make 200ml solutions at half, normal and double the manufacturer's recommended concentration for 6-12 year olds; half a teaspoon (2.5ml), 1 teaspoon (5ml), or 2 teaspoons (10ml) Fybogel Orange (Reckitt Benckiser), respectively, was added (as per manufacturer's instructions) to 200ml blank FaSSIF. The solutions were mixed via manual inversion.

Solutions were made fresh immediately before the first use, then stored at 4°C until stocks were used before making up from fresh again.

2.2 HPLC Methodology

The HPLC (high performance liquid chromatography) machine used consisted of Dionex Ultimate 3000 series pump, Auto Samplers and Diode Array Detectors (Thermo Scientific), and the software used to analyse the samples was Dionex Chromeleon Software Version 7.1 SR1 (Thermo Scientific). Ten μl of each sample was put through an ODS Hypersil C18 column, dimensions: 150mmx4.6mm; particle size: 5 μm (Thermo Scientific), using the appropriate mobile phase below.

For propylthiouracil, mobile phase (50% methanol (Sigma Aldrich); 50% water; v/v) was used with the above column at 1ml/min flow rate for a run time of 5 minutes. UV detection was performed at 276nm. Retention time was approximately 2.2 minutes.

For penicillin V, mobile phase (47% acetonitrile (Fischer Chemical); 3% methanol; 50% 0.01M monobasic potassium phosphate buffer (Sigma Aldrich); v/v/v) was used with the same column at 1.5ml/min flow rate for a run time of 3 minutes. UV detection was performed at 255nm. Retention time was approximately 0.9 minutes.

2.3 Calibration

First, stock solutions of 0.1mg/ml for propylthiouracil powder (Cambridge Biosciences) or 5mg/ml for penicillin V potassium powder (LKT Laboratories) were prepared. Then, a calibration series of 5 different concentrations from this stock solution were prepared (final concentrations of 0.06, 0.07, 0.08, 0.09 and 0.1mg/ml for propylthiouracil; 1, 1.5, 5, 2.5 and 3mg/ml for penicillin V). HPLC analysis was used to measure the drug concentration in 10 μl of each dilution, using the appropriate method in Section 2.2 above. The area under the curve for each

penicillin V sample was recorded, whereas the peak height for each propylthiouracil sample was recorded.

2.3.1 Data Analysis

In Microsoft Excel, the area under the curve (penicillin V) or peak height (propylthiouracil) was plotted against concentration of each dilution. A trend line was fitted to the data using least squares linear regression analysis using the equation $y = mx + c$, where y is area under curve or peak height, x is drug concentration, and m and c are the gradient and intercept of the calibration curve, respectively. The correlation coefficient (R^2) of the calibration curve was also calculated.

2.4 Solubility Measurement

Excess drug powder (1mg propylthiouracil; 5mg penicillin V) was added to a 1.5ml Eppendorf tube, and then 1ml media was added. The tubes were vortexed and then shaken at 37°C for 4 hours within a water bath. Three replicates were prepared for each condition. The tube was centrifuged for 5 minutes at 12,000xg to separate the supernatant, and then the supernatant was removed and used for HPLC analysis, using the appropriate method in Section 2.2 above.

2.4.1 Data Analysis

In Microsoft Excel, the appropriate equation of the trend line from the calibration was used to calculate the drug concentration in each sample from its area under the curve (penicillin V) or peak height (propylthiouracil). The mean and standard deviation of the triplicates was calculated and plotted.

2.5.2 Statistics

In Microsoft Excel, Student's t-tests were only carried out on any samples that had error bars outside of the range of that of blank FaSSIF – the control, reference medium.

2.5 Dissolution

180ml of media was added to a 200ml dissolution vessel, and then one tablet (50mg propylthiouracil (Focus Pharmaceuticals); 250mg penicillin V (Bristol Laboratories) – both supplied by UHB Pharmacy) was added to the vessel. The rotation speed of the paddle was at 75rpm (or 100rpm for the calcium, protein, and fibre solutions). The vessels were incubated at 37°C for 4 hours, with a 1ml sample being taken at fixed time points (0, 15, 30, 45, 60, 120, 180 and 240 minutes) and replaced with 1ml fresh solution to maintain the net volume at 180ml. The samples were then analysed using the appropriate HPLC method in Section 2.2 above.

2.5.1 Data Analysis

In Microsoft Excel, the appropriate equation of the trend line from the calibration was used to calculate the drug concentration in each sample from its area under the curve (penicillin V) or peak height (propylthiouracil). The concentration of each sample was then used to calculate the amount of drug released at that time point. This was used to calculate the amount of drug released as a percentage of the total mass of drug contained in the tablet. The mean and standard deviation of each triplicate was calculated and plotted to show the dissolution of the drug over time.

2.5.2 Statistics

To assess whether there was any statistically significant difference between the dissolution in a particular medium compared to the control, reference medium (blank FaSSIF), the f_1 and f_2 factors [27, 28] were calculated using Microsoft Excel, where f_1 is the difference factor between two dissolution curves, and f_2 is the similarity factor. In order for two dissolution curves to be significantly different, the f_1 value must be above 15 and the f_2 value must be below 50.

3.0 Results

3.1 Summary of Results

In this study, the effect of a range of different food components (pH, fat, calcium, protein and fibre) on the solubility and dissolution profiles of the drugs propylthiouracil and penicillin V was investigated. The different media investigated were: blank FaSSIF, FaSSIF, blank FeSSIF, FeSSIF, and solutions at pH 2.6, pH 4.6, pH 6.6, pH 7.4, 6% fat, 22% fat, 34% fat, 0.2mg/ml Ca^{2+} , 0.5mg/ml Ca^{2+} , 1mg/ml Ca^{2+} , 2mg/ml Ca^{2+} , 2% protein, 4% protein, 6% protein, 8% protein, manufacturer's recommended concentration of fibre for 6-12 year olds, half of this recommended concentration of fibre, and double this recommended concentration of fibre. The results from the different media were compared to that of blank FaSSIF, which acted as a control, reference medium to assess the solubility and dissolution of the two drugs in a fasted state, in the absence of bile salts and fats. Table 4 below summarises the results, showing whether any medium significantly changed the solubility and/or dissolution of either drug compared to that of blank FaSSIF, or not. The results are discussed in detail below.

	FaSSIF/FeSSIF	pH	Fat	Calcium	Protein	Fibre
Propylthiouracil						
Solubility	All solutions ↔	All solutions ↔ ¹	All solutions ↔	All solutions ↔	All solutions ↓	N/A ⁴
Dissolution	Blank FeSSIF and FeSSIF ↓ FaSSIF ↔	pH 6.6 ↓ All other solutions ↔ ¹	22% and 34% fat ↓ 6% fat ↔	2mg/ml Ca ²⁺ ↓ All other solutions ↔	All solutions ³ ↓	N/A ⁴
Penicillin V						
Solubility	All solutions ↔	All solutions ↔ ²	All solutions ↔	All solutions ↔	All solutions ↔	N/A ⁴
Dissolution	All solutions ↔	All solutions ↔ ²	All solutions ↔	All solutions ↔	All solutions ↔ ³	N/A ⁴

¹Did not use media at pH 7.4 for propylthiouracil experiments due to degradation of the drug product in these conditions

²Did not use media at pH 2.6 for penicillin V experiments due to degradation of the drug product in these conditions

³Did not use media at 8% protein for both propylthiouracil and penicillin V dissolution experiments due to interference of casein particles during HPLC analysis

⁴Could not analyse the results for propylthiouracil and penicillin V experiments from any of the fibre media due to uninterpretable data

Table 4 – A summary of the solubility and dissolution of propylthiouracil and penicillin V in a range of different media. The media used are: blank FaSSIF, blank FeSSIF, FaSSIF, FeSSIF, pH 2.6, pH 4.6, pH 6.6, pH 7.4, 6% fat, 22% fat, 34% fat, 0.2mg/ml Ca²⁺, 0.5mg/ml Ca²⁺, 1mg/ml Ca²⁺, 2mg/ml Ca²⁺, 2% protein, 4% protein, 6% protein, 8% protein, half concentration of fibre, normal concentration of fibre, double concentration of fibre. The solubility measurements and dissolution profiles for all other media were compared to the reference media, blank FaSSIF. ↓ indicates a significant reduction in drug solubility/dissolution compared to that in blank FaSSIF; ↔ indicates no significant change in drug solubility/dissolution compared to that in blank FaSSIF.

Table 4 above summaries the solubility and dissolution of propylthiouracil and penicillin V in media containing different levels of various food components (as detailed in the figure legend) compared to the results from the control media, blank FaSSIF. Most media had no significant effect, especially on penicillin V, where none of the results were significantly different from the control. However, all of the media containing different levels of protein caused a significant reduction in the solubility and dissolution of propylthiouracil compared to blank FaSSIF. Other media that caused a significant reduction in the rate of dissolution of propylthiouracil were: blank

FeSSIF, FeSSIF, pH 6.6, 22% fat, 34% fat, and 2mg/ml Ca^{2+} . All other media had no significant effect on propylthiouracil dissolution. Unfortunately, some of the data were uninterpretable due to signal interference (as detailed in the figure legend). The results are discussed in more detail below.

3.2 Solubility of Propylthiouracil

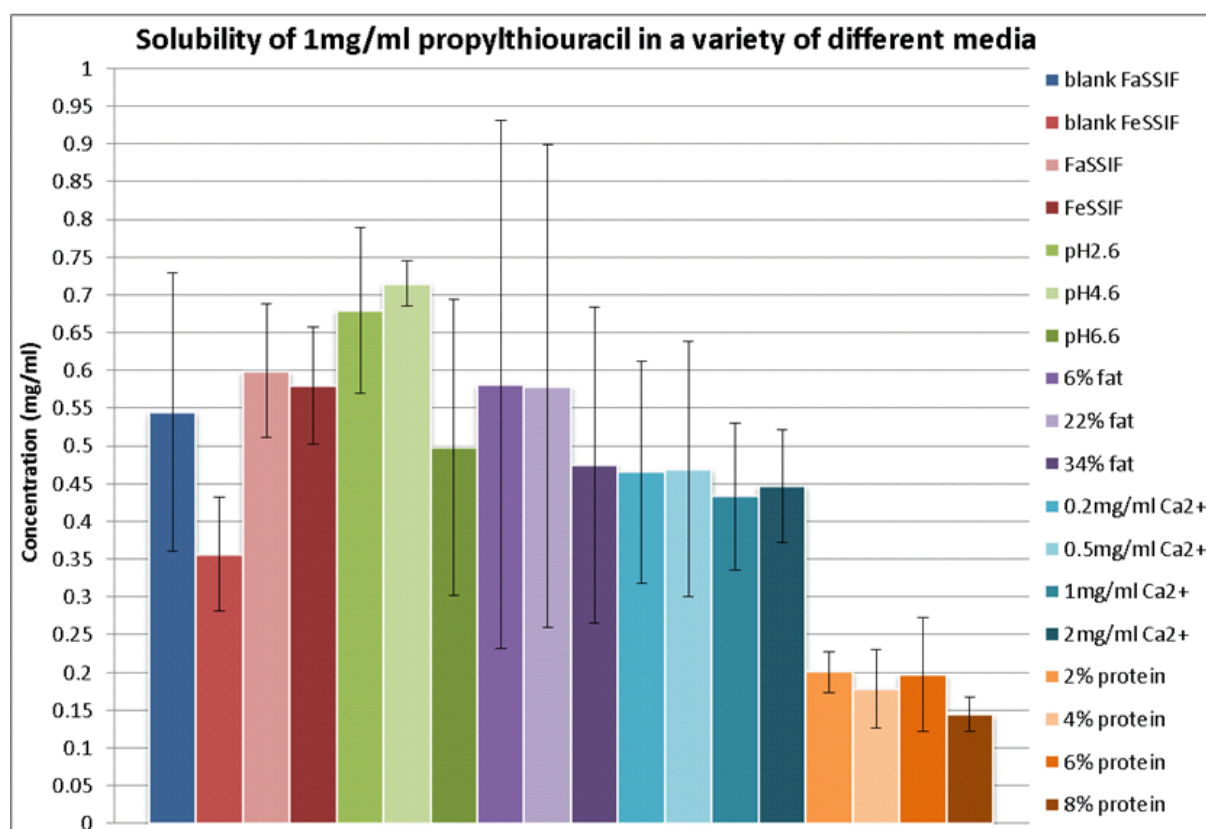


Figure 1 – Summary of the solubility of 1mg propylthiouracil powder in 1ml of media (as indicated in the legend to the right) after 4h incubation at 37°C. Data are the mean concentration ($n = 5$ for blank FaSSIF; $n = 3$ for all other media), error bars are standard deviation.

Figure 1 above shows the solubility of 1mg propylthiouracil powder in 1ml of various media (see figure legend to the right of the graph). The solubility in the control media, blank FaSSIF, (far left) was 0.54 ± 0.18 mg/ml. The only media to cause a significant reduction ($p < 0.05$) in propylthiouracil solubility were the media with varying concentrations of protein (0.02 ± 0.03 for 2% protein; 0.18 ± 0.05 for 4%;

0.20 ± 0.08 for 6%; 0.14 ± 0.02 for 8%). Media at pH 6.6, 34% fat, and all four calcium concentrations also appear to reduce propylthiouracil solubility, although this is insignificant. Blank FeSSIF appears to decrease propylthiouracil solubility even further, but this is still not significant. Conversely, FaSSIF and FeSSIF and media at 6% fat and 22% fat appear to increase the solubility of propylthiouracil, although this is insignificant, particularly as there was a lot of variation between the triplicates in the media at 6% and 22% fat. Media at pH 2.6 and 4.6 appear to increase propylthiouracil solubility even further, but this is still insignificant.

3.3 Dissolution of Propylthiouracil

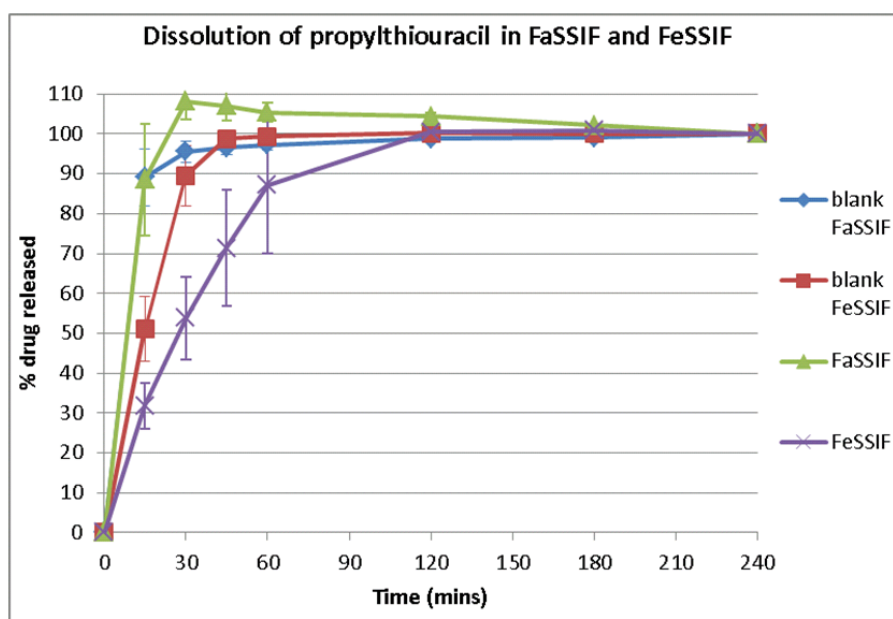


Figure 2 – Dissolution profiles of one 50mg propylthiouracil tablet in 180ml of various media (as indicated in the legend to the right) over 4 hours at 37.4°C. Data are the mean mass of drug released at each time point, expressed as a percentage of the total mass of drug in the tablet (n = 3).

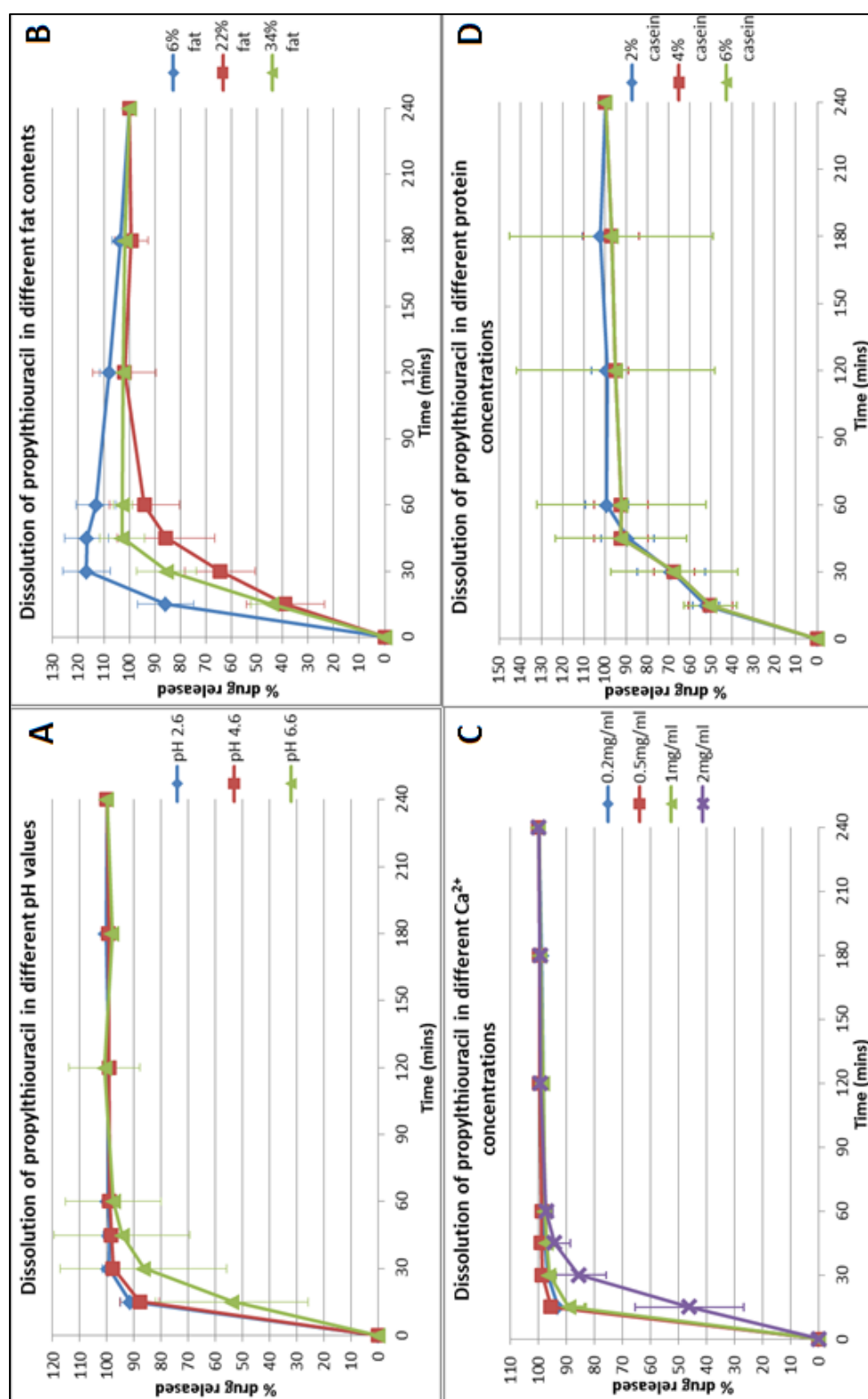


Figure 3 – Dissolution profiles of one 50mg propylthiouracil tablet in 180ml of media with different pH values (A), fat contents (B), calcium concentrations (C) or protein concentrations (D) (as indicated in the legends to the right of each of the graphs) over 4 hours at 37.4°C. Data are the mean mass of drug released at each time point, expressed as a percentage of the total mass of drug in the tablet (n = 3).

Figures 2 and 3 above show the dissolution profiles of propylthiouracil in a variety of media (see figure legends to the right of each of the graphs). Each profile was compared to that of the control media, blank FaSSIF (Fig. 2; blue diamonds). The f_1/f_2 test (see Section 2.5.2) was performed to see if there was a significant difference in the dissolution profile in each medium compared to that of blank FaSSIF. In blank FeSSIF and FeSSIF, the rate of propylthiouracil dissolution was significantly reduced, whereas it was not affected by FaSSIF (Fig. 2). The rate of dissolution of propylthiouracil was also significantly reduced in media at pH 6.6 (Fig. 3A), 22% fat, 34% fat (Fig. 3B), 2mg/ml Ca^{2+} (Fig. 3C), 2% protein, 4% protein, 6% protein, and 8% protein (Fig. 3D). Dissolution was not significantly affected by any of the other media.

3.4 Solubility of Penicillin V

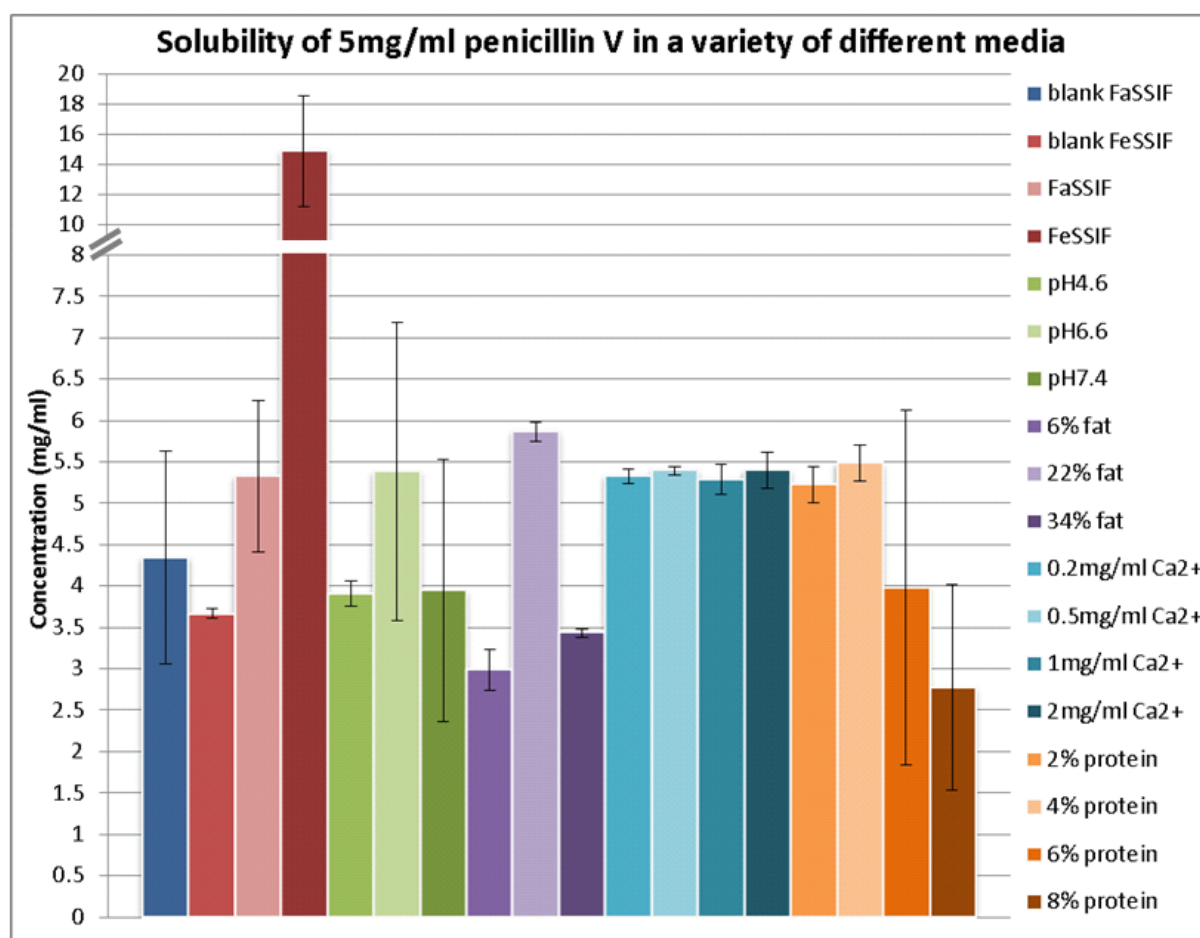


Figure 4 - Summary of the solubility of 5mg penicillin V powder in 1ml of media (as indicated in the legend to the right) after 4h incubation at 37°C. Data are the mean concentration (n = 5 for blank FaSSIF; n = 3 for all other media), error bars are standard deviation.

Figure 4 above shows the solubility of 5mg penicillin V powder in 1ml of various media (see figure legend to the right of the graph). The solubility in the control media, blank FaSSIF, was 4.34 ± 1.28 mg/ml. None of the media had a statistically significant effect, despite FeSSIF and media at 22% fat appearing to give a significant increase in penicillin V solubility. FaSSIF and media at pH 6.6, all four calcium concentrations, 2% protein, and 4% protein, all appear to increase the solubility of penicillin V, although this is insignificant. Blank FeSSIF and media at pH

4.6, pH 7.4, 6% fat, 34% fat, 6% protein, and 8% protein, all appear to reduce the solubility of penicillin V; however, this is not significant.

3.5 Dissolution of Penicillin V

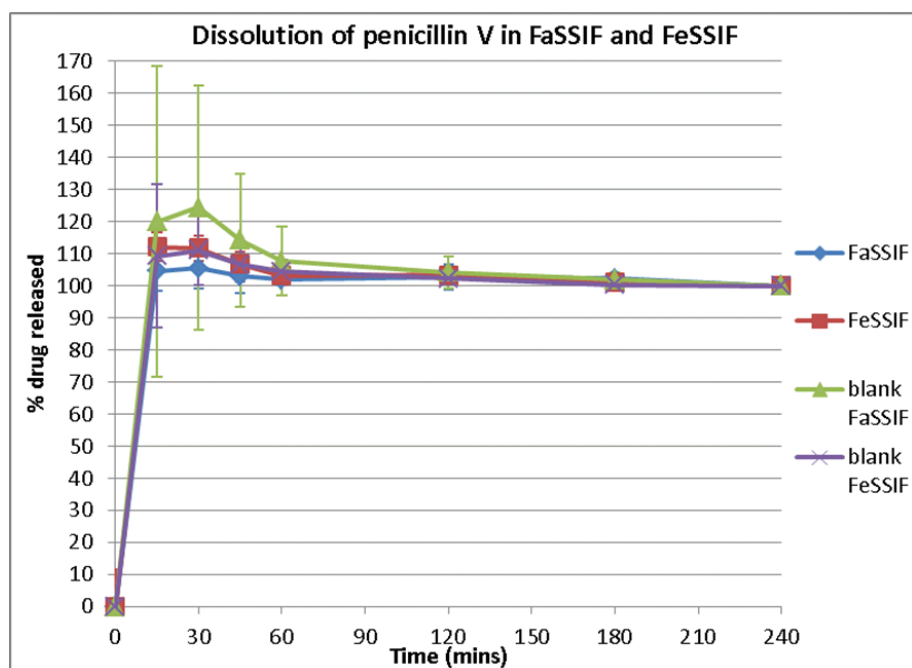


Figure 5 - Dissolution profiles of one 250mg penicillin V tablet in 180ml of various media (as indicated in the legend to the right) over 4 hours at 37.4°C. Data are the mean mass of drug released at each time point, expressed as a percentage of the total mass of drug in the tablet (n = 3).

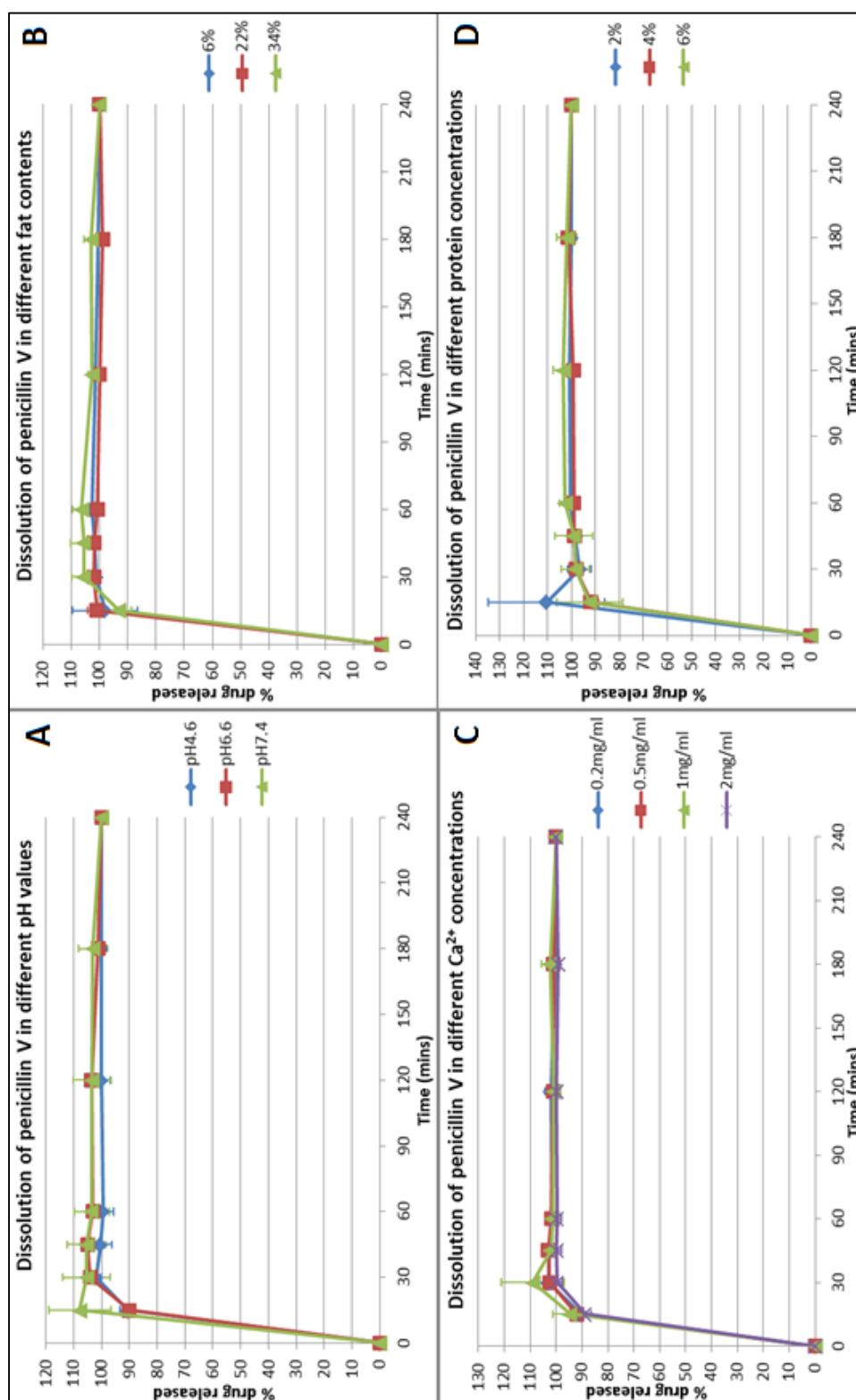


Figure 6 – Dissolution profiles of one 250mg penicillin V tablet in 180ml of media with different pH values (A), fat contents (B), calcium concentrations (C) or protein concentrations (D) (as indicated in the legends to the right of each of the graphs) over 4 hours at 37.4°C. Data are the mean mass of drug released at each time point, expressed as a percentage of the total mass of drug in the tablet (n = 3).

Figures 5 and 6 above show the dissolution profiles of penicillin V in a variety of media (see figure legends to the right of each of the graphs). Each profile was compared to that of the control media, blank FaSSIF (Fig. 5; blue diamonds). The f_1/f_2 test (see Section 2.5.2) was performed to see if there was a significant difference in the dissolution profile in each medium compared to that of blank FaSSIF. However, the rate of dissolution of penicillin V was not significantly affected by any of the media, as at least 90% penicillin V was released from the tablet within the first 15 minutes in all of the media.

4.0 Discussion

4.1 Effect of fasted and fed states

Compared to blank FaSSIF (the control, reference fasted state media, which does not contain bile salts or fats), both FaSSIF and FeSSIF caused a slight increase in the solubility of propylthiouracil, whereas blank FeSSIF resulted in a decrease its solubility (Fig. 1), although none of these changes were significant. Conversely, both blank FeSSIF and FeSSIF significantly reduced the dissolution profile of propylthiouracil, but FaSSIF had no effect (Fig. 2). While this data is mixed, it would seem that the solubility and dissolution of propylthiouracil is reduced in the fed state, which has been shown previously [20].

While there was no significant difference in the rate of dissolution of penicillin V in blank FeSSIF, FaSSIF or FeSSIF compared to blank FaSSIF (Fig. 5), its solubility appeared to be affected, albeit insignificantly (Fig. 4). FaSSIF resulted in a slight increase in solubility, whereas blank FeSSIF resulted in a slight decrease in solubility. FeSSIF seemed to cause a massive increase in penicillin V solubility to around 15mg/ml. However, this cannot be believed as, while FeSSIF does contain

various fats and surfactants which can increase drug solubility, this is a three-fold increase in drug concentration from the initial 5mg of penicillin V powder added to 1ml media (see Section 2.4), which seems unlikely to be achieved by the contents of FeSSIF alone. It is unclear what caused this very large difference – it is too big to be human error, so it might have been an error in the HPLC machine, for example, it may have had some residual drug in it, as this sample was run after running the fibre media through it (this media was difficult to analyse using the current HPLC method – for more details, see Section 4.6). Consequently, this experiment needs repeating in order to determine the real solubility of penicillin V in FeSSIF, as time constraints prevented this from occurring. Similar to propylthiouracil, despite the data being mixed, it appears that the solubility and dissolution of penicillin V is also reduced in the fed state, which has been shown before [16, 20].

Consequently, it would be best to advise that both propylthiouracil and penicillin V be given on an empty stomach and without food, in order to maintain optimal drug efficacy. This is what is currently advised, however, as previously discussed, this is rarely possible in paediatrics. In the sections below are details of the effects of each of the specific food components on the solubility and dissolution of both drugs to see which components have the greatest effect, in order to best advise both parents and medical professionals which foods and drinks to avoid when giving either drug to children.

4.2 Effect of pH

The data from propylthiouracil solubility in pH 7.4 media, and penicillin V solubility in pH 2.6 media, were uninterpretable due to interference with the HPLC signal (Table 4). It would seem that this was due to instability and degradation of the drug products

in these media [29], which meant that it was difficult to identify which peak represented the intact drug product, as desired, and which were a result of degradation. Consequently, the dissolution experiment was not carried out for these drugs in their respective media.

The rate of dissolution of propylthiouracil was significantly reduced in pH 6.6 media, but was unaffected by media at lower pH (Fig. 3A). Furthermore, the solubility appeared to be reduced in pH 6.6 media, but increased in media at lower pH, although this is insignificant (Fig. 1). This, combined with the instability and degradation of the drug product in pH 7.4 media (Table 4), suggests that propylthiouracil is less stable at more neutral pH values. It would be interesting to see if propylthiouracil stability is affected at higher pH, as this would show if it is the fact that the drug is more stable at lower pH, or just unstable at neutral pH. Either way, this is less likely to be problematic in practice, as few foods are within the alkaline pH range, and the physiological pH of the digestive system tends to be lower than neutral. However, this is more likely to have an effect in infants, whose diets tend to be comprised of milk, which has a pH of around 6.7 [30, 31]. Consequently, it would be best to advise parents to avoid giving their child propylthiouracil around the time of feeding them milk.

The solubility and dissolution of penicillin V was not significantly affected by any of the pH media (Figs. 4 and 6A), indicating that pH has no effect on penicillin V. However, as previously stated, the data from its solubility in media at pH 2.6 was unreadable, most likely due to degradation of the drug product [29]. Furthermore, its solubility in media at pH 4.6 was $3.90 \pm 0.15\text{mg/ml}$, compared to $4.34 \pm 1.28\text{mg/ml}$ in blank FaSSIF. This shows that the solubility of penicillin V is reduced at lower pH, and at low pH, the drug product itself becomes broken down by the acidic

environment [29]. Consequently, it would be best to advise parents to avoid giving their child penicillin V with acidic foods and drinks because it would reduce the drug's efficacy. This is particularly important to stress, as parents often give the crushed tablet mixed with apple sauce, or dissolved in fruit juices, which have a pH of around 3.4 [31], and thus is likely to cause degradation of the drug product. Conversely, Fig. 4 shows that penicillin V solubility was also reduced in media at pH 7.4, implying that the drug's solubility may also be affected by more neutral pH. However, as the error bars are quite large, this effect is questionable and requires more repeats to investigate this further, as time constraints prevented this from occurring.

4.3 Effect of fat

Propylthiouracil solubility was not significantly affected by fat (Fig. 1); however there was a large amount of variation between the triplicates, indicating that the experiments may need to be repeated to confirm this, as time constraints prevented this from occurring. In contrast, its dissolution profile was significantly different in higher fat contents, suggesting that fat reduces the rate of dissolution of propylthiouracil. This indicates that parents should be advised against giving this drug with high-fat foods and drinks, as it is likely to reduce its efficacy. However, children's diets are quite high in fat because, unlike adults – whose energy is derived mostly from carbohydrate, children (infants in particular) obtain most of their energy from the calories in fat [24] (see Table 3). Moreover, milk is a large component of the diet, especially in babies and infants, and yoghurt is another common foodstuff that drugs are mixed with before being given to a child [25, 26], and these foods can be quite high in fat [30, 32]. Consequently, fat can have quite a large impact on the efficacy of drugs in children. However, as nutritional contents are now readily

available on product packaging, it is possible to purchase the foodstuffs with the lowest fat content – this is likely to be good advice to parents, as they will have the option to still use these commonly used foodstuffs when administering drugs to their children, whilst improving the efficacy of the drug.

While Fig. 6B shows that the dissolution of penicillin V was not significantly affected by fat, Fig. 4 shows a mixed effect of fat on penicillin V solubility, although this is not significant. Both 6% and 34% fat media resulted in a decrease in solubility, suggesting that fat reduces the solubility of penicillin V, which has been seen previously [16, 17]. However, the decrease is larger in the 6% media, which is unexpected. Opposing this, 22% fat media resulted in an increase in solubility to nearly 6mg/ml, which is greater than the 5mg of penicillin V powder added to 1ml of media (see Section 2.4), suggesting that this result is unreliable and that it needs to be repeated, as time constraints prevented this from occurring. However, this media was made from infant formula milk, rather than normal milk powder like the other two media, meaning that more powder was needed to make the 22% media, subsequently making this media much thicker, which may have interfered with the HPLC readings. Consequently, it would be worth repeating this experiment with media from more comparable sources to make the results more reliable, as time and resource constraints prevented this from occurring. From this data, no conclusions can be drawn on the effect of fat on penicillin V, but it is advisable to warn parents to try and avoid giving penicillin V with high-fat foods and drinks, by either administering with an alternative foodstuff, or purchasing lower-fat variants, in order to improve the efficacy of the drug.

4.4 Effect of calcium

Fig. 1 shows that calcium reduced propylthiouracil solubility, but in a concentration-independent manner. However, this is not a significant effect. Additionally, Fig. 3C shows that only media at 2mg/ml calcium caused a significant reduction in the rate of propylthiouracil dissolution. As calcium only altered the dissolution profile at high concentrations, and only had a small, statistically insignificant effect on solubility, it seems that calcium content of the foods or drinks given with propylthiouracil is not a great concern to the efficacy of the drug, as long as the calcium levels are not too high. However, children's diets are quite high in calcium, as milk is a large component of the diet, especially in babies and infants. Furthermore, yoghurt is another common foodstuff that drugs are mixed with before being given to a child. As dairy products are high in calcium [30, 32], for example, 100g milk and 100g fruit-flavour yoghurts designed for children both contain approximately 120µg calcium [30, 32], it is worth making parents aware that giving propylthiouracil with milk or yoghurt can reduce the drug's efficacy.

Fig. 6C shows that the dissolution profile of penicillin V was unaffected by the presence of calcium. Conversely, Fig. 4 seems to show that calcium causes an increase in penicillin V solubility, again in a concentration-independent manner, although this is not significant. However, we must be cautious with this data, despite it having very tight error bars, because the concentrations of penicillin V in all four media are slightly over 5mg/ml (the initial concentration of penicillin V powder when added to the media – see Section 2.4). Consequently, these experiments should be repeated to see if this is an effect of calcium, or inaccurate weighing, as time constraints prevented this from occurring. These data suggest that the efficacy of

penicillin V is unaffected by calcium, so foods and drinks high in calcium need not be avoided when making penicillin V more palatable for children, although this requires further investigation.

However, in the calcium media, there was an increasing amount of visible solid particles suspended in solution as calcium concentration increased. As a result, these particles might have been taken up into the HPLC machine, thus affecting the reading of the drug concentration. Consequently, the data in Fig. 3C showing that the rate of dissolution of propylthiouracil was significantly reduced in media at 2mg/ml Ca^{2+} may not be true. However, as the dissolution of penicillin V in media at 2mg/ml Ca^{2+} was not affected, nor was the dissolution of either drug affected in media at lower calcium concentrations, we can be more confident that this data is true. Still, it may be best to repeat the experiment, using filters on the syringes when sampling in order to remove these particles, as time constraints prevented this from occurring. This would allow the HPLC machine to more accurately measure the drug concentration of each sample, without the risk of signal interference from calcium chloride particles.

4.5 Effect of protein

As shown in Figs. 1 and 3D, both the solubility and dissolution of propylthiouracil were significantly reduced in all of the protein media ($p < 0.05$). However, there does not seem to be a definite concentration-dependent effect, as the solubility in the four different protein media is variable, and there is no significant difference between the dissolution profiles in the three protein media investigated. This suggests that protein affects propylthiouracil independently of its concentration. However, it may be worth

investigating this further with more repeats, as a slight trend can be seen, but time constraints preventing this from happening.

Conversely, neither the solubility nor the dissolution of penicillin V was significantly affected by the protein media, suggesting that protein has no significant effect on penicillin V. However, Fig. 4 does show decreased penicillin V solubility with increased protein concentration. Further repeats may reveal that this is a significant effect, as time constraints prevented this from occurring, but it may be that protein only has a significant effect on penicillin V solubility at higher concentrations than those investigated here, and thus are less likely to be physiologically relevant. Despite this, it is still worth investigating the effect of higher protein concentrations on the solubility and dissolution of penicillin V, but time constraints prevented this from happening.

As milk and yoghurts are also high in protein [30, 32], it may be best to advise that parents avoid giving either drug with these foodstuffs or around the time of consuming them, in order to prevent the protein affecting the absorption of the drug. However, as previously stated, it is now possible to purchase the foodstuffs with the lowest protein content due to nutritional information on food packaging, meaning that parents still have the option to still use these commonly used foodstuffs when administering drugs to their children whilst improving the efficacy of the drug.

Unfortunately, no results were obtained from the dissolution of either drug in the 8% protein media. This was because of strong signal interference, resulting in unreadable data. Like the calcium media, the protein media had some visible solid particles suspended in solution, which increased with increased protein concentration. It may have been that these particles entered the HPLC machine,

causing the measurement of drug content to be reduced, rather than a true effect of the protein concentration on drug dissolution. Similarly, the use of filters on the syringes during sampling would allow this to be investigated further, but time constraints prevented this from happening.

As a consequence of these particles being present, the results from Figs. 1 and 3D showing that propylthiouracil solubility and dissolution were significantly reduced in the protein media may just be an artefact, due to signal interference from the large casein particles suspended in solution in all four of the different protein media. Again, use of syringes would help to show if this was a true effect. However, as the solubility and dissolution of penicillin V was not significantly changed in the protein media, we can be more confident that the observed effect of protein on propylthiouracil solubility and dissolution is true.

4.6 Effect of fibre

As stated in Table 4, there were no results obtained from any of the experiments carried out in the fibre media because the data was unreadable. This could be due to one of several reasons. It may be that the visible particles suspended in the Fybogel media were taken up into the HPLC machine and thus affected the results, in a similar way to the results from the protein media and higher calcium concentrations might have been affected. Likewise, the way to investigate whether this is the case is to use filters on the syringe when sampling, as time constraints prevented this from happening. Alternatively, it may be that the fibre media were too viscous to be suitable for the current dissolution protocol. Despite increasing the rotation speed to 100rpm, it may be that any dissolved drug was unable to be evenly dispersed throughout the media, resulting in unreadable data. Furthermore, it may be that, in

the current HPLC set up, the HPLC machine was unable to take up the same amount of fibre media each time. Consequently, the HPLC methodology would have to be optimised to consistently take up 10µl of a more viscous solution. However, time constraints prevented this from happening. In spite of this, the problem may lie within the media used itself, for example, preventing adequate dispersion of the dissolved drug product. If this is the case, an alternative method for investigating the effect of fibre would need to be found, as time and resource constraints preventing this from being investigated.

4.7 Improvements to penicillin V dissolution experiments

From these experiments, it would seem that the rate of dissolution of penicillin V is unaffected by any food components (Figs. 5 and 6). However, it may be that food components do affect the dissolution profile, but at a more subtle level than the above methods detailed in Section 2.5 can detect. Consequently, it may be worth modifying the protocol to take samples every 5 minutes for 30 minutes, for example, to see if there are any significant differences during this period, as time constraints prevented this from being carried out. These differences would have less of an impact on drug efficacy than those detected within the normal sampling range, as they are more subtle, but are still worth examining.

4.8 Further work

In this project, the *in vitro* effect of these food components on the solubility and dissolution of propylthiouracil and penicillin V was investigated. It would be beneficial to then investigate if the *in vivo* effect is similar, by conducting fed effect clinical studies in children, looking at a range of meal compositions, differing in pH, and the content of fat, calcium, protein and fibre, in order to support these results.

It would also be useful to investigate whether any effect can be observed in various commonly used food stuffs, such as yoghurt, milk, and apple sauce, both *in vitro* and *in vivo*, in order to confirm these results.

4.9 Conclusions

These data show that the absorption of propylthiouracil would be most affected by foodstuffs with high fat, calcium and protein contents, and higher pH. Consequently, it would be best to advise parents against giving this drug to children with milk or yoghurt, for example. However, propylthiouracil may be administered with foods such as apple sauce or fruit juice, as this will have less of an effect on its oral absorption.

Less conclusive data is available from these experiments on which food components significantly affect penicillin V absorption. However, it would be best to advise parents against giving this drug to children with apple sauce or fruit juices, as low pH seems to degrade the drug. Alternatively, penicillin V may be administered with foods like milk or yoghurt, as calcium and protein seem to have less of an effect on the oral absorption of penicillin V. However, as fat may have a negative effect on the absorption of penicillin V, it may be best to recommend administering with low fat milk or yoghurt in order to improve drug efficacy.

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Project 2:

**AN INVESTIGATION INTO THE ROLE
OF OESTROGEN RECEPTOR β IN
MEDULLARY THYROID CANCER**

This project is submitted in partial fulfilment of
the requirements for the award of the MRes

Abstract:

Medullary thyroid cancer (MTC) is a rare, aggressive tumour type. Most hereditary and half of sporadic MTC cases are caused by activating mutations in the Ret proto-oncogene. However, hereditary MTC has recently been found to be associated with inactivating mutations in oestrogen receptor β (ER β). Oestrogen acting through ER β initiates anti-proliferative signalling pathways in order to restrain cell growth. When functional ER β is lost through mutation, this regulation may be lost, potentially resulting in tumourigenesis. The aims of this project were to see if significant ER β knockdown could be achieved in SW1736 and MCF7 cell lines, and whether this knockdown has any effect the expression of Ret, cyclin D1 and IGF-1R (other oestrogen-responsive genes involved in cell proliferation). ER β knockdown was carried out by transfecting specific siRNAs. RNA and protein expression were determined by quantitative-PCR and Western blotting, respectively. Significant ER β knockdown was achieved at the protein level in SW1736 cells, and at the RNA level in MCF7 cells. In ER β knocked-down MCF7 cells, cyclin D1 RNA levels trended towards decreased expression, whereas IGF-1R RNA levels trended towards increased expression in the presence of oestrogen. From these data, it is unlikely that MTC arises due to pro-proliferative changes in Ret or cyclin D1 expression, as RNA levels of these genes were statistically unchanged when levels of functional ER β were significantly reduced, or when oestrogen was present. However, these data do suggest that MTC might arise due to increases in IGF-1R expression in cases where expression of functional ER β is reduced.

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List of Abbreviations

E2	– 17 β -oestradiol (oestrogen)
EGFR	– epidermal growth factor receptor
ERK	– extracellular signal-related kinase
ER α	– oestrogen receptor α
ER β	– oestrogen receptor β
ESR2	– oestrogen receptor β gene
FMTC	– familial medullary thyroid cancer
IGF-1R	– insulin-like growth factor type 1 receptor
JNK	– c-Jun N-terminal kinases
MAPK	– mitogen-activated protein kinase
MEN2A	– multiple endocrine neoplasia type 2A
MEN2B	– multiple endocrine neoplasia type 2B
MTC	– medullary thyroid cancer
Negative	– MCF7 cells transfected with the negative control siRNA, and not treated with oestrogen
Negative + E2	– MCF7 cells transfected with the negative control siRNA, and treated with 10nM oestrogen
PI3K	– phosphatidylinositol 3'-kinase
Ret	– rearranged during transfection proto-oncogene
siRNA	– MCF7 cells transfected with the combination of two anti-ESR2 siRNAs (s4826 and s4827), and not treated with oestrogen
siRNA + E2	– MCF7 cells transfected with the combination of two anti-ESR2 siRNAs (s4826 and s4827), and treated with 10nM oestrogen
siRNA 1	– anti-ESR2 siRNA s4826 (Ambion)
siRNA 2	– anti-ESR2 siRNA s4827 (Ambion)
siRNA 3	– anti-ESR2 siRNA s4828 (Ambion)

VEGFR

– vascular endothelial growth factor receptor

1.0 Introduction

1.1 Medullary thyroid cancer

Medullary thyroid cancer (MTC) is a rare tumour of the parafollicular C cells in the thyroid gland [1-5]. Thyroid cancer makes up approximately 1% of all cancers worldwide [6], and MTC is responsible for approximately 5% of all thyroid cancers [2, 3, 5, 7, 8]. Generally, MTC is a more aggressive form of thyroid cancer [7], with metastases to lungs, liver and bone occurring early [2], and prognosis becoming worse with increased age and/or more advanced stage at diagnosis [7].

MTC is sporadic in 75% of cases. In the other 25% of cases, MTC is hereditary, in the form of either multiple endocrine neoplasia (MEN) 2A, 2B, or familial (FMTC) [2-5, 7, 8]. MEN2A and 2B are also often associated with other neoplasms, such as pheochromocytoma (tumours of the adrenal medulla) [3]. While MEN2A usually presents in the third decade of life if it is not detected through genetic screening (see Section 1.7), MEN2B tends to be more aggressive and usually presents very early – often in the first year of life [3]. Conversely, FMTC is rarely associated with any other neoplasm, and is less aggressive than MEN, typically presenting in the fourth decade of life [3]. The gene that is mutated in hereditary MTC and half of sporadic cases of MTC is Ret [7, 9-12].

1.2 Ret signalling

1.2.1 Structure

The rearranged during transfection (Ret) proto-oncogene was found in 1985 to be located on chromosome 10q11.2 [11-13]. The gene encodes a receptor tyrosine kinase, which is 170kDa in size and found on the cell surface [9, 11, 13]. Alternative

splicing of the gene results in three main isoforms: Ret9, Ret49, and Ret51, also referred to as the short-, medium- and long-domains, respectively [9, 12]. The protein domains of Ret are shown below in Fig. 1.



Figure 1 – Protein domains of the rearranged during transfection (Ret) receptor tyrosine kinase proto-oncogene. The N-terminal (N) extracellular domain is responsible for ligand binding, and consists of four repeats of cadherin-like motifs (blue) and a cysteine-rich domain (red). The transmembrane domain (TD) is a short, hydrophobic region (purple). The C-terminal (C) intracellular region consists of a tyrosine kinase domain, with several tyrosine residues (green). This is the functional domain, which becomes activated upon ligand binding and is responsible for activating downstream signalling cascades. (Adapted from [7, 9, 11])

As shown in Fig. 1, the intracellular tyrosine kinase domain located towards the C terminus of the Ret protein is activated when the ligand binds to the extracellular domain (found towards the N terminus of the protein), and is responsible for activating the various downstream signalling cascades [7, 9, 11], as shown in Fig. 2 below.

1.2.2 Signalling

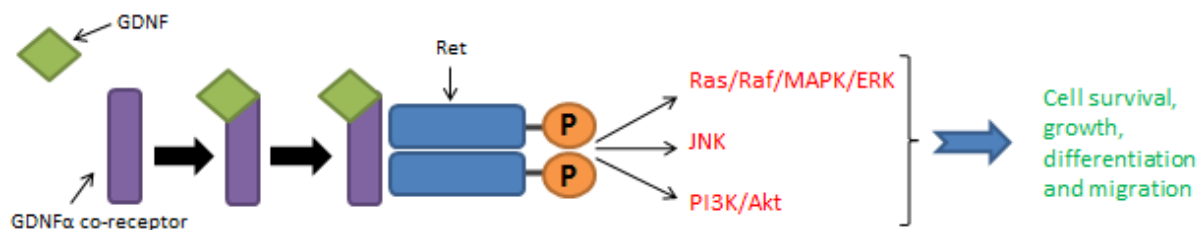


Figure 2 – Activation pathway of the rearranged during transfection (Ret) receptor tyrosine kinase proto-oncogene. Glial cell derived neurotrophic factor (GDNF; green diamond) binds to a member of the GDNF receptor α (GDNF α) family (purple rectangle). Ret (blue rectangle) homodimerisation results in autophosphorylation of the tyrosine kinase domains (orange circles labelled 'P'). The three main signalling pathways downstream of activated Ret are: Ras/Raf/mitogen-activated protein kinase (MAPK)/extracellular signal-related kinase (ERK); c-Jun N-terminal kinases (JNK); phosphatidylinositol 3'-kinase (PI3K)-Akt, which all promote cell survival, growth, differentiation and migration. (Adapted from [4, 7, 9, 11, 12])

The endogenous ligand of Ret is glial cell derived neurotrophic factor (GDNF), which is a member of the neuronal growth factor family. Ret is activated by GDNF binding to a glycosylphosphatidylinositol (GPI)-anchored co-receptor from the GDNF receptor α (GDNF α) family, as shown in Fig. 2. This results in the homodimerisation of Ret, which facilitates autophosphorylation of the tyrosine kinase domains, thus activating Ret [7, 9, 11, 12]. Once Ret is activated, it can then stimulate various downstream signalling cascades. The three main cascades promoted by Ret are the Ras/Raf/mitogen-activated protein kinase (MAPK)/extracellular signal-related kinase (ERK) pathway, the c-Jun N-terminal kinases (JNK) pathway, and the phosphatidylinositol 3'-kinase (PI3K)-Akt pathway [4, 7, 12]. Activation of these pathways results in cell growth, survival, migration and differentiation [7, 9].

1.3 The role of Ret mutations in cancer

As activated Ret is responsible for promoting cell growth, survival and migration, when Ret is mutated to become constitutively active, there is uncontrolled cell proliferation, resulting in the formation of tumours.

As previously stated in Section 1.1, Ret is often mutated in MTC. Activating Ret mutations in the germline are responsible for hereditary forms of MTC [3-5, 7, 8]. In MEN2A, Ret tends to be mutated within the cysteine-rich domain [3, 4], whereas the Ret mutation most commonly seen in MEN2B is M918T, which is associated with an increased risk of metastases, thus resulting in a decreased survival rate and worse prognosis [3, 4, 7]. Furthermore, somatic Ret activating mutations cause approximately half of all sporadic cases of MTC [3-5, 7, 8]. This shows that Ret is an important factor in the initiation and development of MTC.

1.4 Other factors that may be involved in medullary thyroid cancer

Several different genes have also been found to be altered in MTC, and consequently may be useful targets for MTC therapies.

Various growth factors and their receptors are known to be involved in MTC. It has been found that vascular endothelial growth factor receptor (VEGFR) and epidermal growth factor receptor (EGFR) are often overexpressed in MTC [3-5, 14]. Insulin-like growth factor type-1 receptor (IGF-1R) can also be overexpressed in MTC [12].

Activation of these receptors and their subsequent downstream signalling pathways can result in the initiation and progression of MTC, due to enhanced tumour growth (through increased cell proliferation and survival) and invasion [7, 12].

Another factor recently found to be altered in MTC is oestrogen receptor β (ER β). A number of patients with hereditary MTC but lacking a Ret mutation have been discovered to have a mutation in the ER β gene [unpublished observation].

Furthermore, normal parafollicular C cells express ER, and transformed cells maintain the ability to synthesise ER [1], suggesting that oestrogen signalling is important in malignant parafollicular C cells.

1.5 Oestrogen receptor signalling

1.5.1 Structure

Oestrogen (17 β -oestradiol; E2) is a steroid hormone known to regulate a vast range of cellular processes, including cell survival, proliferation and differentiation, across the whole body – not just in the male and female reproductive systems [15].

Oestrogen signalling is most commonly mediated by the binding of oestrogen to the oestrogen receptor (ER) – a nuclear receptor (see Section 1.5.2). There are two

isoforms of the ER: ER α and ER β . While the ER α gene is located on chromosome 6q25.1 and encodes a 66kDa protein [16], and the ER β gene is located on chromosome 14q23.2 and encodes a 56kDa protein [17], they both have a similar modular protein structure [18], as shown in Fig. 3 below.



Figure 3 – Protein domains of the oestrogen receptor (ER). At the N-terminus (N), there is the ligand-independent activation function-1 (AF-1). The C domain (red) contains the DNA binding domain (DBD). The D domain contains the nuclear localisation signal (NLS). Towards the C-terminus (C), there is the ligand binding domain (LBD) and the ligand-dependent activation function-2 (AF-2). (Adapted from [15, 18, 19])

Fig. 3 shows the modular structure of ER α and ER β . The N-terminal A/B domain contains the ligand-independent activation function-1 (AF-1). The C domain contains the DNA binding domain (DBD), which allows the ER to bind to oestrogen response elements (EREs) in DNA. The D domain is also known as the hinge region, as it can rotate to give different conformations of ER. This domain also contains the nuclear localisation signal (NLS). The E domain contains both the ligand-dependent activation function-2 (AF-2) and the ligand binding domain (LBD), which is where oestrogen can bind to the ER. Furthermore, this domain can interact with other ERs, resulting in homo- and heterodimerisation, and with heat-shock proteins and other chaperone proteins. However, the functions of the C-terminal F domain are still unclear [15, 18, 19]. The ER α and ER β proteins have conserved DBD and LBD regions, allowing both receptors to bind to oestrogen and EREs, but they have different N-terminal structures, resulting in different biological responses to oestrogen (see Section 1.5.2) [6, 20]. However, both receptors are activated in the same way, as shown below.

1.5.2 Signalling

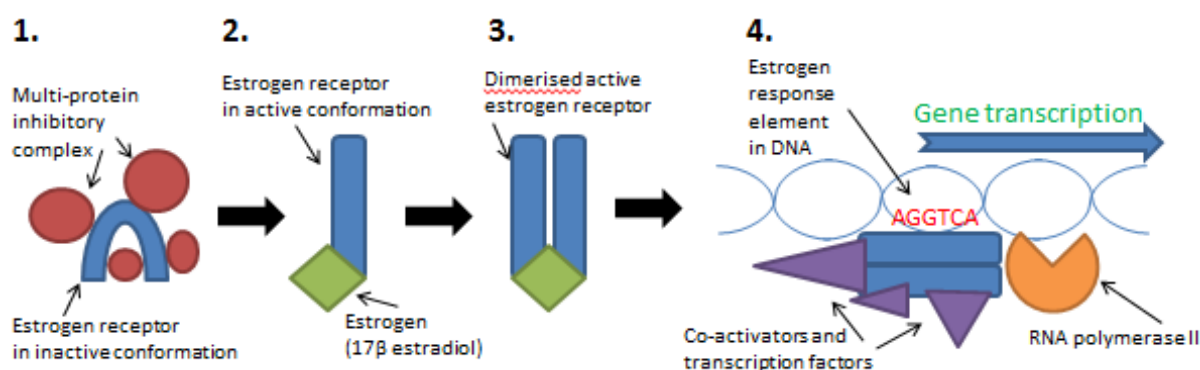


Figure 4 – The classical (genomic) pathway of oestrogen receptor (ER) signalling. When oestrogen (green diamond) is absent, ER (blue) is bound within a multi-protein inhibitory complex (red circles). Activated ER binds to oestrogen receptor elements (ERE) in the DNA, along with various co-activators and transcription factors (purple triangles), and RNA polymerase II (orange) to regulate transcription of target genes. (Adapted from [6, 15, 18, 20-22])

The main mechanism of ER signalling is the classical, or genomic, pathway, as shown in Fig. 4. In the absence of oestrogen, the ER is sequestered by chaperone proteins in the nucleus, such as heat-shock proteins, maintaining ER in an inactive form within the inhibitory complex [15, 21, 22]. When oestrogen binds to the ER, there is a conformational change within the ER, which facilitates dissociation of the chaperone proteins and dimerisation of ER. It has been shown that heterodimerisation can occur, as well as homodimerisation, resulting in slightly different biological responses. This dimerisation allows the ER to bind to the DNA at specific EREs, which have a conserved DNA sequence of AGGTCA [6, 15, 20, 21]. The ER also recruits various co-factors to the transcription start site, such as co-activators, co-repressors, transcription factors, and RNA polymerase II, in order to regulate transcription of the target genes [6, 15, 18]. These co-factors are responsible for either modulating the interaction between the ER and the transcription machinery, or remodelling the chromatin structure. Various co-activators possess histone acetyltransferase, which acetylates N-terminal lysine

residues of histones, thus neutralising the positive charge of the lysine residues, which decreases the affinity of histones for the negatively-charged DNA. This opens up the DNA structure, facilitating binding of transcription machinery to gene promoter regions [15]. Conversely, various co-repressors possess histone deacetylase, which reverses histone acetylation, resulting in a more compact DNA structure, thus preventing the binding of the transcription machinery [15].

However, this is not the only mechanism of ER signalling. A membrane-bound ER, the G protein coupled receptor GP30, has recently been found [21, 23]. Signalling through this novel receptor results in the activation of various signalling cascades, such as the MAPK and ERK pathways, or the release of intracellular calcium [6, 15, 18, 21, 22]. This is a much more rapid form of oestrogen signalling, as there is a much shorter lag time between oestrogen binding and signalling output, because there is no transcription or translation required [15].

Another method of ER signalling is known as transcription factor cross-talk, in which activated ERs interact with various transcription factors, such as activating protein-1 (AP-1) and stimulating protein 1 (SP1) [18]. Activated ERs have been found to activate or repress genes that are responsive to these transcription factors in different ways, depending on the cell type, receptor and promoter [15].

There is also a ligand-independent pathway of ER signalling. Activation of growth factor receptors, such as EGFR and IGF-1R, results in the stimulation of various signalling cascades, which can result in the activation of ER or its co-receptors, even in the absence of ligand [18, 19, 21]. Furthermore, the phosphorylation status of ER, and therefore its activity, can be regulated depending on the balance of kinases and phosphatases that act on the ER [24].

It has been observed that ER α and ER β have different expression profiles, as well as different biological functions, as illustrated by the distinct phenotypes of ER α knockout animals compared to ER β knockout animals, suggesting that they play different roles in oestrogen signalling in health and disease (for more details, see Section 1.6) [6, 18, 20, 22, 23]. Not only do the two isoforms regulate the activity of each other due to the competition for binding to EREs [6, 20], but ER β has been found to inhibit ER α activity through decreasing the sensitivity of ER α to oestrogen, and through promoting ER α degradation [6, 18, 20]. This shows that ER β is also critical for regulating oestrogen signalling itself, indicating that this is an important regulatory protein.

1.6 The role of oestrogen receptors in cancer

Oestrogen has been found to play a role in several different types of cancer, namely breast, ovarian, endometrial, colonic and prostate cancers [18], and has recently been thought to be involved in MTC (see Section 1.4). The main way in which oestrogen signalling is altered in cancer is through an increased ER α :ER β ratio (i.e. increased expression of ER α and/or decreased expression of ER β) [2]. It has been shown that oestrogen signalling through ER α promotes tumour initiation and progression by increasing cell growth, while signalling through ER β opposes tumour development by preventing cell growth [2, 21]. ER α and ER β also have different influences on the expression of cyclin D1 [18], a key protein in the regulation of the cell cycle, as it facilitates progression from the G₁ phase to the S phase [6, 21, 25-27]. Oestrogen signalling through ER α increases the expression of cyclin D1, thus promoting cell proliferation, whereas oestrogen signalling through ER β decreases cyclin D1 expression, thus inhibiting cell proliferation [2, 18]. Additionally, when

thyroid cancer cell lines were treated with the ER α agonist propyl-pyrazole-triol (PPT), there was increased cell proliferation and expression of the anti-apoptotic protein B cell lymphoma-2 (Bcl-2), but when the cell lines were treated with the ER β agonist diarylpropionitrile (DPN), cell proliferation was inhibited and Bcl-2 expression was reduced [6, 21]. Furthermore, tumours that still express ER β are associated with a better prognosis because the tumour tends to be much less aggressive [2]. This shows that ER β has a protective, anti-proliferative effect, which is often lost in cancers, resulting in unregulated cell proliferation, and thus tumour progression.

1.7 Treatment of medullary thyroid cancer

MTC does not respond to conventional cancer therapies, such as chemotherapy or radiation [2, 4, 5]. Therefore, the main form of treatment for MTC is total thyroidectomy [2, 4, 5], although response to this has been found to be improved by local lymph node dissection [4, 5]. Due to the severity of the disease, it is recommended that genetic screening of relatives of MTC patients is carried out, and that total thyroidectomy is undertaken in all those with activating Ret mutations as a prophylactic treatment, due to the aggressive nature of MTC [3].

However, there is now a drug treatment for metastatic MTC. These treatments involve the use of multiple tyrosine kinase inhibitors to target the signalling cascades responsible for promoting cell proliferation, survival and migration (see Section 1.4) [3-5, 7, 14]. The first United States Food and Drug Administration (US FDA) approved drug for the treatment of MTC was vandetanib, in April 2011 [4, 5, 7]. Vandetanib inhibits Ret, VEGFR and EGFR [3-5, 7, 14], and in clinical trials increased progression-free survival in patients with metastatic MTC from 9.3 to 30.5 months [14]. However, vandetanib is not without its problems. Common side effects

include diarrhoea, nausea, headaches, rashes, and hypertension, but it was also found to significantly prolong the QT interval on electrocardiograms, thus increasing the risk of developing the fatal heart condition Torsades de pointes [3, 4, 7]. An alternative drug for treating metastatic MTC is cabozantinib, which inhibits Ret, VEGFR and hepatocyte growth factor receptor (HGFR) [7, 14], and in clinical trials increased progression-free survival in patients with severe metastatic MTC from 4.0 to 11.2 months [14]. However, the toxicology profile of cabozantinib is still high, with 90% of patients experiencing at least one side effect – the most common being gastrointestinal disturbances [7].

Consequently, there is a demand to develop novel treatments that are more successful at treating MTC, with fewer adverse effects. In order to achieve this, we need greater understanding of the signalling processes behind MTC so that we can find new targets for therapeutics. By increasing our knowledge of the role ER β plays in MTC, we may be able to target this signalling pathway and develop a new treatment for MTC.

1.8 Aims and hypotheses

It has been observed that there is an increased ER α :ER β ratio in MTC [21]. Furthermore, mutations in the ER β gene have been found in a number of patients with hereditary MTC who lack a mutation in the Ret gene [unpublished observation], supporting the idea that mutated ER β results in the initiation and/or progression of cancer. This thesis investigates the hypothesis that ER β mutations results in Ret hyperactivity, through disruption of ER β -mediated Ret inhibition, and that this is a key pathway involved in MTC development these cases.

In order to do this, the first aim was to achieve successful (i.e. more than 70%) knockdown of ER β by transfecting anti-ESR2 siRNA into two different cell lines: SW1736 (a human thyroid carcinoma cell line) and MCF7 (a human breast carcinoma cell line). Both of these cell lines have been confirmed previously by the McCabe lab to be oestrogen-responsive (so contain both ER α and ER β) and express Ret, as shown by quantitative-PCR (q-PCR).

The next aim was to see if successful knockdown of ER β had any significant effect on the level of RNA expression of Ret in SW1736 and MCF7 cells, using q-PCR.

The final aim was to discern the effect of treating ER β -knockdown MCF7 cells with 17 β -oestradiol (E2) on the amount of RNA expression of ER β , Ret and the oestrogen-responsive genes cyclin D1 and insulin-like growth factor type-1 receptor (IGF-1R), via q-PCR.

2.0 Materials and Methods

2.1 Cell culture

The cell lines SW1736 (human thyroid carcinoma) and MCF7 (human breast carcinoma) were obtained from The American Type Culture Collection. Both cell lines were maintained at 37°C and 5% CO₂ in complete RPMI 1640 media [+] L-glutamine from Gibco (containing 10% foetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (Pen/Strep; Gibco)) in 75cm³ Corning flasks. When the cell lines reached 90% confluency, usually twice a week, they were sub-cultured at a 1:6 or 1:10 dilution, respectively. The medium was aspirated from the cells, washed with PBS (5x phosphate buffered saline tablets (Sigma) in 500ml dH₂O), trypsinised (0.025% Trypsin/EDTA, Invitrogen) for approximately 1 minute at 37°C, and then

transferred to new 75cm³ flasks containing fresh complete RPMI medium at approximately 37°C in the cell densities described.

For transfection, both cell lines were seeded at 60,000 cells/well in a 12-well plate for subsequent RNA extraction, and 150,000 cells/well in a 6-well plate for subsequent protein extraction, and maintained at 37°C and 5% CO₂.

2.2 Transfection

24 hours after seeding, cells were transfected with 50nM or 100nM either AllStars negative control siRNA (Qiagen) or anti-ESR2 *Silencer*® Select Pre-designed siRNA (s4826, s4827 or s4828; Ambion) using HiPerFect transfection reagent (Qiagen) according to manufacturer's instructions. SW1736 cells were transfected with 50nM or 100nM negative siRNA or one of the three anti-ESR2 siRNAs. MCF7 cells were transfected with 100nM negative siRNA or a combination of the s4826 and s4827 anti-ESR2 siRNAs. Each experimental condition for RNA transfection was repeated four times, while each experimental condition for protein transfection was repeated twice.

Transfection mixes were prepared as follows:

For RNA extraction of SW1736 cells, 12µl HiPerFect and either 6µl 10µM siRNA + 82µl Opti-MEM reduced serum media (Gibco); or 12µl 10µM siRNA + 76µl Opti-MEM, were mixed to give 100µl of transfection mix at 50nM or 100nM siRNA, respectively, for one well.

For RNA extraction of MCF7 cells, 12µl HiPerFect and either 12µl 10µM negative siRNA + 76µl Opti-MEM; or 6µl of each 10µM anti-ESR2 siRNA + 76µl Opti-MEM, were mixed to give 100µl of transfection mix at 100nM siRNA, for one well.

For protein extraction of SW1736 cells, 24µl HiPerFect and either 12µl 10µM siRNA + 64µl Opti-MEM; or 24µl 10µM siRNA + 52µl Opti-MEM, were mixed to give 100µl of transfection mix at 50nM or 100nM siRNA, respectively, for one well.

For protein extraction of MCF7 cells, 24µl HiPerFect and either 24µl 10µM negative siRNA + 52µl Opti-MEM; or 12µl of each 10µM anti-ESR2 siRNA + 52µl Opti-MEM, were mixed to give 100µl of transfection mix at 100nM siRNA, for one well.

Transfection mixes were vortexed, incubated for 10 minutes at room temperature, and vortexed again before 100µl was added drop-wise to each respective well. Each condition was replicated four times for RNA extraction, and twice for protein extraction. Cells were incubated at 37°C and 5% CO₂.

2.3 Oestrogen treatment

17β-oestradiol (Sigma Aldrich) was resuspended in ethanol to 10mM, and stored at -20°C for up to a month. MCF7 cells were sub-cultured at a 1 in 10 dilution into 75cm³ Corning flasks containing complete charcoal-stripped, clear RPMI 1640 media (Gibco) containing 10% FBS and 1% Pen/Strep (as in Section 2.2). Cells were incubated for 48 hours at 37°C and 5% CO₂ before seeding into 12-well and 6-well plates in charcoal-stripped media at the densities specified in Section 2.1 above. 24 hours after seeding, cells were transfected using the transfection mixes above. 24 hours after transfection, the 10mM 17β-oestradiol was diluted to 5µM with water. Then, 5µM 17β-oestradiol was added to each well, to give a final concentration of 10nM oestrogen. After 6 hours of oestrogen treatment, cells in the 12-well plates were harvested; the cells in the 6-well plates were harvested after 24 hours of oestrogen treatment.

2.4 RNA extraction using RNeasy MicroKit

24 hours after transfection, medium was aspirated from the wells; cells were washed with PBS, and 250µl TRI-reagent (Sigma) was added to each well. After 5 minutes incubation at room temperature, this was transferred into a clean 1.5ml Eppendorf tube and stored at -80°C overnight.

Once samples were thawed the next day, 50µl chloroform (Sigma) was added to each tube, and then the tubes were inverted 30 times before being left to stand at room temperature for 15 minutes. Samples were centrifuged at 13,200rpm for 20 minutes at 4°C, and 100µl of the clean, top, clear layer was transferred to a clean 1.5ml Eppendorf. Then, 125µl 100% ethanol (Sigma) was added to each tube and samples were vortexed before being transferred to a separate column from the RNeasy® Micro Kit (Qiagen), as per manufacturer's instructions. These instructions were followed to complete the RNA extraction into 14µl RNase-free water (Qiagen). The RNA concentration of each sample was measured using the NanoDrop.

2.5 Reverse transcription polymerase chain reaction (RT-PCR)

Using the RNA concentrations of each sample as determined above, each sample was diluted using RNase-free water (Qiagen) to give 200ng or 400ng RNA in 5µl for the SW1736 and MCF7 cell lines, respectively. Samples were incubated at 70°C for 10 minutes, span down and put on ice. A master mix from the reagents in the Reverse Transcription System (Promega) was prepared as per manufacturer's instructions, and 5µl of this was added to each sample. Two negative control samples were also run during each experiment. Samples were incubated at room temperature for 15 minutes before being incubated at 42°C for 1 hour, then 95°C for 5 minutes, and then 4°C for 5 minutes. Samples were centrifuged at 13,200rpm for

30 seconds and 10µl of RNase-free water was added to each sample before being stored at -20°C.

2.6 Quantitative polymerase chain reaction (q-PCR)

PCR master mixes were prepared as follows: 7µl RNase-free water + 10µl 2x GoldStar Taqman PCR master mix (Eurogentec) + 1µl 20x Taqman gene expression assay (Applied Biosystems) – this is for one well. To assay for the desired genes ESR2, Ret, cyclin D1, IGF-1R and the control gene 18S, the following Taqman assays were used: Hs00230957_m1 ESR2, Hs01120030_m1 RET, Hs00765553_m1 CCND1, Hs00609566_m1 IGF1R, and QuantumRNA™ classic 18S internal standard respectively. The 18S Taqman assay uses a Vic reporter and a TAMRA quencher, while all the other Taqman assays use a FAM reporter and a TAMRA quencher.

To each assigned well of a 96-well plate, 2µl and 18µl of the appropriate sample and master mix, respectively, was added. Each sample was run on the plate in duplicate. The plate was sealed thoroughly with a film lid, centrifuged at 2,000rpm for 30 seconds, and ran on a Taqman PCR machine.

2.6.1 Data Analysis

In Microsoft Excel, delta C_T values and fold changes were calculated from the C_T values for each sample and gene assayed. The mean C_T values and fold change for each experimental condition was plotted, with error bars representing standard deviation for delta C_T values, and standard error of the mean for fold changes. Student's t-test was performed using Microsoft Excel to test for significance ($p < 0.05$).

2.7 Protein extraction

48 hours after transfection, medium was aspirated from the wells, cells were washed with PBS, and 150µl RIPA/PI/PhI solution was added to each well. This solution was made up of 1ml RIPA lysis buffer : 60µl protease inhibitor cocktail (Sigma) : 10µl Halt™ phosphatase inhibitor cocktail (Thermo Scientific). The RIPA lysis buffer was made in house and stored at 4°C, and was composed of 1.22g Trizma base (Sigma) + 1.8g sodium chloride (Fischer Scientific) + 160ml dH₂O + 2ml Igepal (Sigma) + 5ml 10% sodium deoxycholate (Sigma) + 2ml 100mM EDTA (3.722g in 100ml; Sigma), adjusted to pH7.4 with HCl. Cells were incubated at -20°C for 15 minutes, and then each well was scraped and transferred to a 1.5ml Eppendorf tube. Samples were sonicated on medium for 30 seconds, before centrifuging at 12,000rpm for 10 minutes at 4°C. The supernatant was transferred to a fresh 1.5ml Eppendorf tube and the protein concentration of each sample was measured using the BCA assay below, and the samples were stored at -20°C.

2.8 BCA assay

To each assigned well of a 96-well plate, 4µl of each sample and protein standards (0, 0.125, 0.5, 0.75, 1, 1.5, 2, 2.5 and 5mg/ml protein in RIPA buffer) was added in duplicate. Then, 80µl of BSA reagent (1ml reagent A : 20µl reagent B (Pierce® BCA assay, Thermo Scientific)) was added to each well. The plate was then incubated at 37°C for 30 minutes. Protein concentration was then measured using a spectrophotometer and the programme Wallac Victor 3, using the 'Absorbance 560' protocol.

2.9 Western blotting

Samples were incubated with protein sample buffer (0.107g DL-dithiothreitol (DTT; Sigma) + 1ml Laemmli sample buffer (Biorad)) for 5 minutes at 95°C before being loaded, along with molecular weight markers (Precision plus protein dual colour standards, Biorad), into a well of a 12% gel. The gel was made in house on Day 1 of the Western blot and comprised a 12% resolving gel, which was poured into the mould and left to set for 20 minutes, and a 5% stacking gel, which was poured into the mould on top of the set resolving gel. The comb was inserted into the stacking gel. The 12% resolving gel was composed of 2.4675ml dH₂O + 1.875ml 1.5M Tris-HCl at pH 8.8 (90.75g Trizma base + 500ml dH₂O) + 3ml acrylamide (Protogel; National Diagnostics) + 75µl 10% sodium dodecyl sulphate (SDS; Sigma) + 75µl 10% ammonium persulphate (APS; Sigma) + 7.5µl tetramethylethylenediamine (TEMED; Sigma). The 5% stacking gel was composed of 1.51ml dH₂O + 0.625ml 0.8M Tris-HCl at pH 6.8 (48.5g Trizma base + 500ml dH₂O) + 312.5µl acrylamide + 50µl 10% SDS + 50µl 10% APS + 5µl TEMED. Samples were ran with 1x running buffer (made in house and stored at room temperature – 10x running buffer, consisting of 30g Trizma base + 144g glycine (Sigma) + 10g SDS in 1L dH₂O, was diluted to 1x with dH₂O) at 140V for approximately 40 minutes, and then transferred to a PVDF nitrocellulose membrane (Amersham Hybond P PVDF membrane; GE Life Sciences) using transfer buffer (made in house and stored at room temperature – comprised of 6.06g Tris + 28.8g glycine + 400ml methanol + 1.6L dH₂O) at 360mA for 75 minutes. Membranes were then blocked in TBST (made in house and stored at room temperature – comprised of 50ml 1M Tris at pH 7.6 (121.14g Trizma base in 1L dH₂O) + 20g 5mM sodium chloride + 0.625ml Tween-80 (Sigma) + 2449.4ml dH₂O) + either 5% milk (Marvel) or 5% BSA (bovine serum albumin; Sigma) at room

temperature for at least 1 hour before being incubated at 4°C overnight in TBST + 5% milk or BSA containing rabbit anti-ER β primary antibody (ER β (H-150): sc-8974; Santa Cruz) at a dilution of 1:200. Membranes were washed with TBST (three times five minute washes) before being incubated at room temperature for 1 hour in TBST + 5% milk containing goat anti-rabbit secondary antibody (Dako) at a dilution of 1:2,000. Membranes were washed with TBST (three times five minute washes) before being incubated in Pierce® ECL2 Western Blotting substrate (Thermo Scientific), as per manufacturer's instructions, and then developed onto medical X-ray film (Kodak) in the dark.

As a control, membranes were re-probed for β -actin to ensure even protein loading. To do this, membranes were washed with TBST (three times five minute washes) before being blocked in TBST + 5% milk at room temperature for at least 1 hour before being incubated at 4°C overnight in TBST + 5% milk containing murine anti- β actin primary antibody (Sigma) at a dilution of 1:10,000. Membranes were washed with TBST (three times five minute washes) before being incubated at room temperature for 1 hour in TBST + 5% milk containing rabbit anti-murine secondary antibody (Dako) at a dilution of 1:2,000. Membranes were washed with TBST (three times five minute washes) before being incubated and developed as above.

2.9.1 Data Analysis

The X-ray film was scanned and the file opened in Image J 1.45s software. Band density was analysed using pixel numbers in the allocated band area. In Microsoft Excel, the fold change for each experimental condition was calculated compared to the negative condition. Student's t-test was performed using Microsoft Excel to test for significance ($p < 0.05$).

3.0 Results

3.1 Data for SW1736 cells transfected with control or anti-ESR2 siRNA

3.1.1 Quantitative-PCR

3.1.1.1 ER β

SW1736 cells (a human thyroid carcinoma cell line) were transfected with either a negative control siRNA or one of three different anti-ESR2 siRNAs in order to see if significant knockdown of ER β could be achieved at the RNA level in this cell line. Fig. 5 below shows the level of ER β RNA expression in SW1736 cells transfected with either a negative control siRNA or one of three different siRNAs targeted against the ESR2 gene. Fig. 5A shows the mean delta C_T values of ER β RNA expression in each experimental group, with error bars representing standard deviation (SD). In the negative siRNA-transfected group, the mean delta C_T value was 23.9 ± 0.2 , which remained statistically unchanged when transfected with siRNA 1 (s4826), siRNA 2 (s4827), or siRNA 3 (s4828). Fig. 5B shows the mean fold change in ER β RNA expression compared to the expression of the control gene 18S, with error bars representing standard error of the mean (SEM). In the negative siRNA-transfected group, the mean fold change was 1.0 ± 0.1 , which remained statistically unchanged in the three experimental conditions. However, the cells transfected with siRNA 3 trended towards a knockdown of ER β RNA in Figs. 5A and 5B, which was trending towards significance ($p = 0.42$ and 0.27 , respectively, compared to between 0.8 and 0.9 for the other experimental conditions).

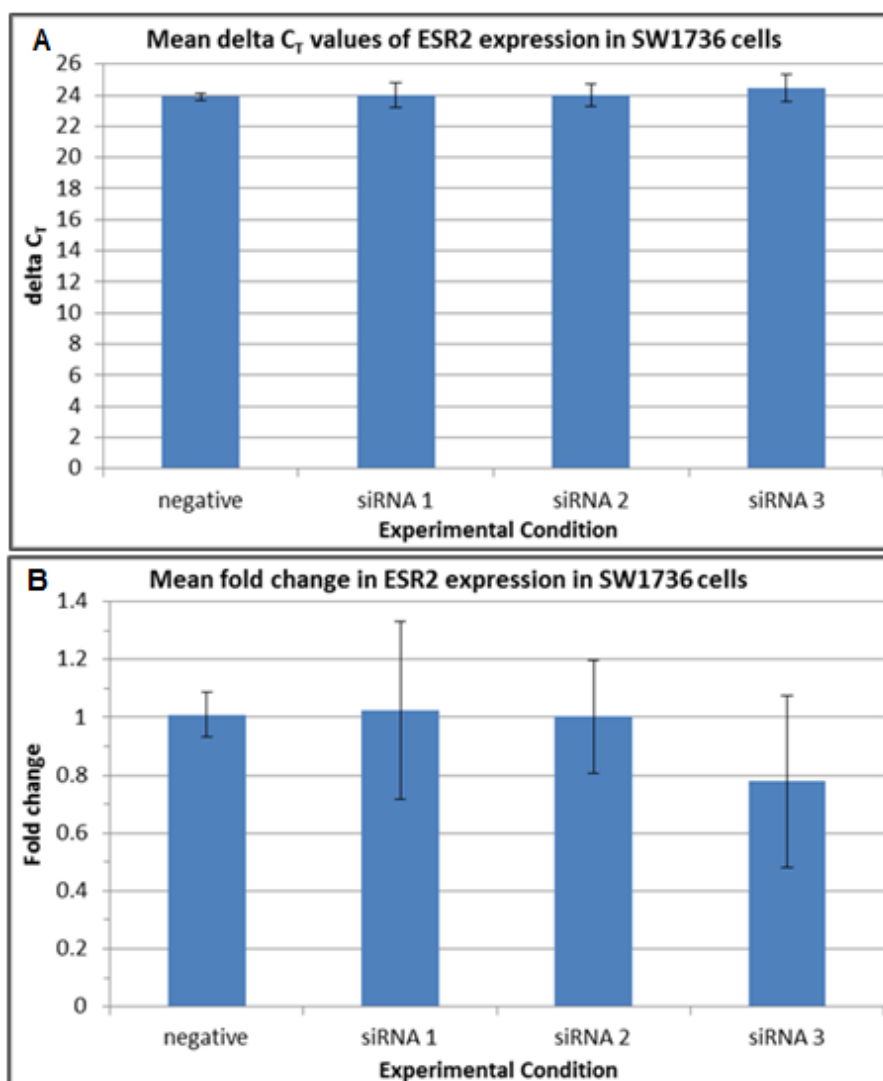


Figure 5 – Levels of oestrogen receptor β (ER β) RNA expression in SW1736 cells transfected with either a negative control siRNA or one of three siRNAs targeted against ESR2, in terms of delta C_T values (A) and fold change (B). Data were obtained from q-PCR and are mean values ($n = 3$ for the siRNA 2 and siRNA 3 experimental groups; $n = 4$ for the negative and siRNA 1 groups), with error bars showing standard deviation (A) and standard error of the mean (B).

3.1.1.2 Ret

The SW1736 cells that had been transfected with a negative control siRNA or one of three different anti-ESR2 siRNAs were also assayed for RNA expression of Ret in order to see if the level of ER β RNA expression had any effect on the level of Ret RNA expression in this cell line. Fig. 6 below shows the level of Ret RNA expression in SW1736 cells transfected with either a negative control siRNA or one of three

different siRNAs targeted against the ESR2 gene. Fig. 6A shows the mean delta C_T values of Ret RNA expression in each experimental group, with error bars representing SD. The mean delta C_T value for the negative siRNA-transfected group was 26.6 ± 0.3 , which was statistically unchanged in the groups transfected with any anti-ESR2 siRNA. However, the delta C_T value of the cells transfected with siRNA1 trended towards an increase to 27.1 ± 0.4 in the group treated with siRNA 1, which was approaching significance ($p = 0.09$). Fig. 6B shows the mean fold change of Ret RNA expression, with error bars representing SEM. The mean fold change in the negative siRNA-transfected group was 1.0 ± 0.1 , which remained statistically unchanged in all experimental groups. However, the fold change trended towards an increase in Ret RNA expression in the siRNA 2-transfected group to 1.3 ± 0.2 , and trended towards a decrease in Ret RNA expression in the siRNA 1-transfected group to 0.7 ± 0.1 . Both of these changes were approaching significance ($p = 0.30$ and 0.10 , respectively).

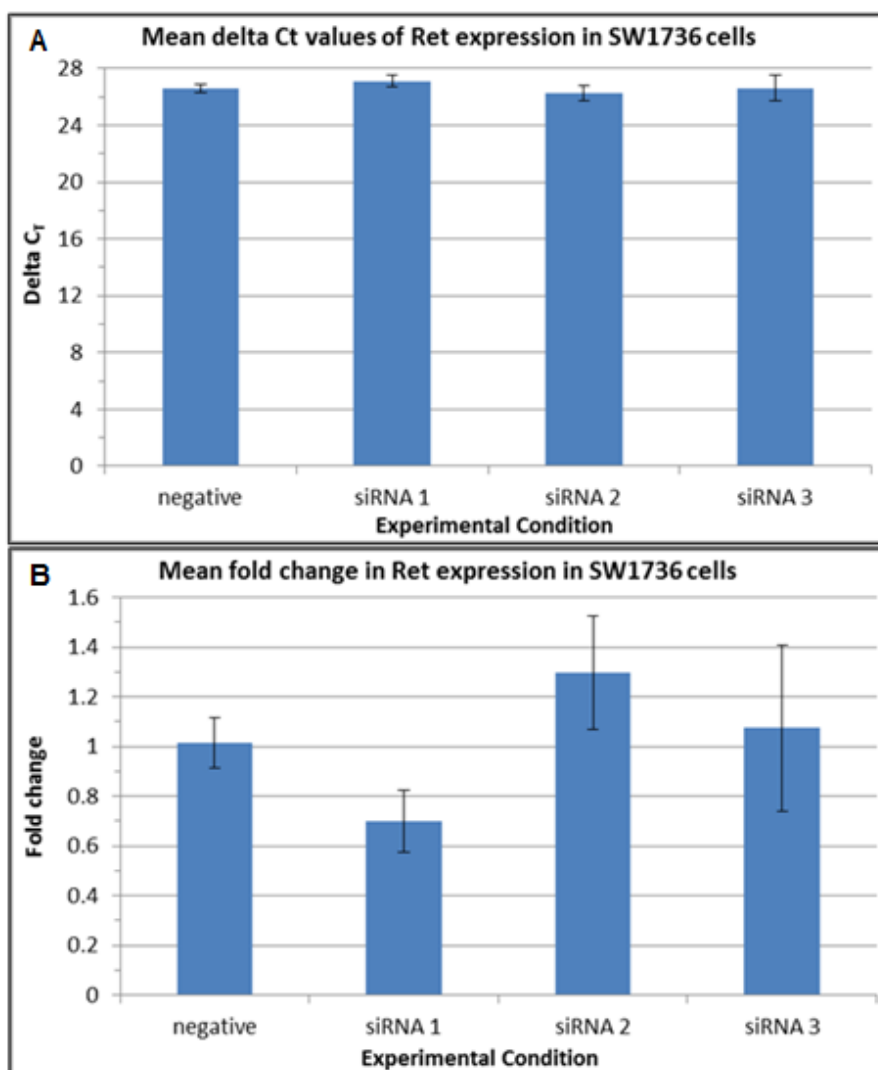


Figure 6 – Levels of Ret RNA expression in SW1736 cells transfected with either a negative control siRNA or one of three siRNAs targeted against ESR2, in terms of delta C_T values (A) and fold change (B). Data were obtained from q-PCR and are mean values ($n = 3$ for the siRNA 2 and siRNA 3 experimental groups; $n = 4$ for the negative and siRNA 1 groups), with error bars showing standard deviation (A) and standard error of the mean (B).

3.1.2 Western blot

SW1736 cells were transfected with either a negative control siRNA or one of three different anti-ESR2 siRNAs in order to see if significant knockdown of ER β could be achieved at the protein level in this cell line. Figs. 7 and 8 below are repeats of the same experiment, so contain the same protein samples, but only 20 μ g of protein was loaded into each well in Fig. 7, and this membrane was blocked with milk and exposed to X-ray film for 30 minutes, whereas 40 μ g of protein was loaded into each

well in Fig. 8, and this membrane was blocked with BSA and exposed to X-ray film for 1 minute, due to high levels of background staining. ER β was detected around 55kDa, while β -actin was detected around 50kDa, and bands were analysed using densitometry (see Section 2.9.1). Fig. 7A shows the level of ER β protein expression in SW1736 cells transfected with either a negative control siRNA or one of three different siRNAs targeted against the ESR2 gene. The two lanes containing samples transfected with the negative siRNA showed a clear band at 55kDa. This band was significantly weaker in the lanes containing samples transfected with siRNA 1 (s4826) and siRNA 2 (s4827), with the bands almost non-existent in the siRNA 1-transfected group ($p < 0.05$). The bands trended towards being weaker in the lanes containing samples transfected with siRNA 3 (s4828), although this was not significant. Fig. 7B shows the level of β -actin protein expression in each experimental group. The much darker β -actin bands in these lanes in Fig. 7B suggests more protein was loaded in these lanes, whereas the bands in other lanes showed a more even protein loading. Fig. 8A also shows the level of ER β protein expression in SW1736 cells transfected with either a negative control siRNA or one of three different siRNAs targeted against the ESR2 gene. The band densities remained statistically unchanged in all experimental conditions, despite trending towards weaker bands in each anti-ESR2 siRNA-transfected group. Fig. 8B shows fairly consistent bands of β -actin in each lane, showing even protein loading.

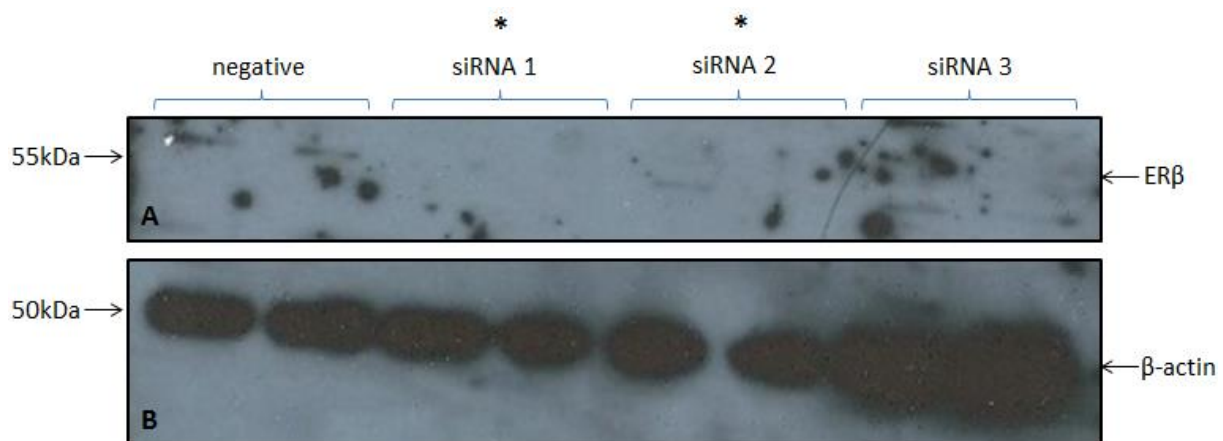


Figure 7 – Western blot showing oestrogen receptor β (ER β) protein expression (A) and β -actin protein expression (B) in SW1736 cells transfected with either negative control siRNA or one of three siRNAs targeted against ESR2. Amount of protein loaded was 20 μ g, with 30 minute exposure to X-ray film. * = $p < 0.05$ (Student's t-test)

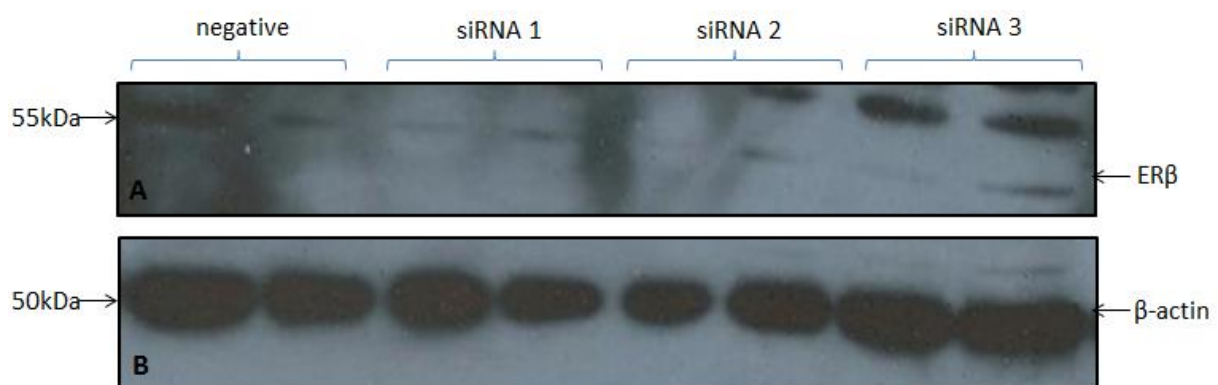


Figure 8 – Western blot showing oestrogen receptor β (ER β) protein expression (A) and β -actin protein expression (B) in SW1736 cells transfected with either negative control siRNA or one of three siRNAs targeted against ESR2. Amount of protein loaded was 40 μ g, with 1 minute exposure to X-ray film.

3.2 Data for MCF7 cells transfected control or anti-ESR2 siRNA, and treated with or without oestrogen

3.2.1 Quantitative-PCR

3.2.1.1 ER β

MCF7 cells (a human breast carcinoma cell line) were transfected with either a negative control siRNA or a combination of two different anti-ESR2 siRNAs (s4826

and s4827) in order to see if significant knockdown of ER β could be achieved at the RNA level in this cell line. Furthermore, these transfected cells were then treated either with or without 10nM oestradiol (E2) in order to see if the presence of oestrogen significantly affected the level of expression of ER β RNA. Fig. 9 below shows the level of ER β RNA expression in MCF7 cells transfected with either a negative control siRNA or a combination of different siRNAs against ESR2 (s4826 and s4827), and then either treated with or without 10nM oestradiol (referred to as negative, negative + E2, siRNA, and siRNA + E2 in the rest of this thesis). Fig. 9A shows the mean delta C_T values of ER β RNA expression in each experimental group, with error bars representing SD. The mean delta C_T value for the negative group was 19.8 ± 0.3 , which was statistically unchanged in the negative + E2 and siRNA + E2 groups. However, the mean C_T value was significantly increased to 20.4 ± 0.3 in the siRNA group ($p < 0.05$). Fig. 9B shows the mean fold change in ER β RNA expression, with error bars representing SEM. The mean fold change in the negative group was 1.0 ± 0.1 , which was statistically unchanged in the negative + E2 and siRNA + E2 groups. Conversely, the mean fold change was significantly decreased to 0.6 ± 0.1 in the siRNA group ($p < 0.05$).

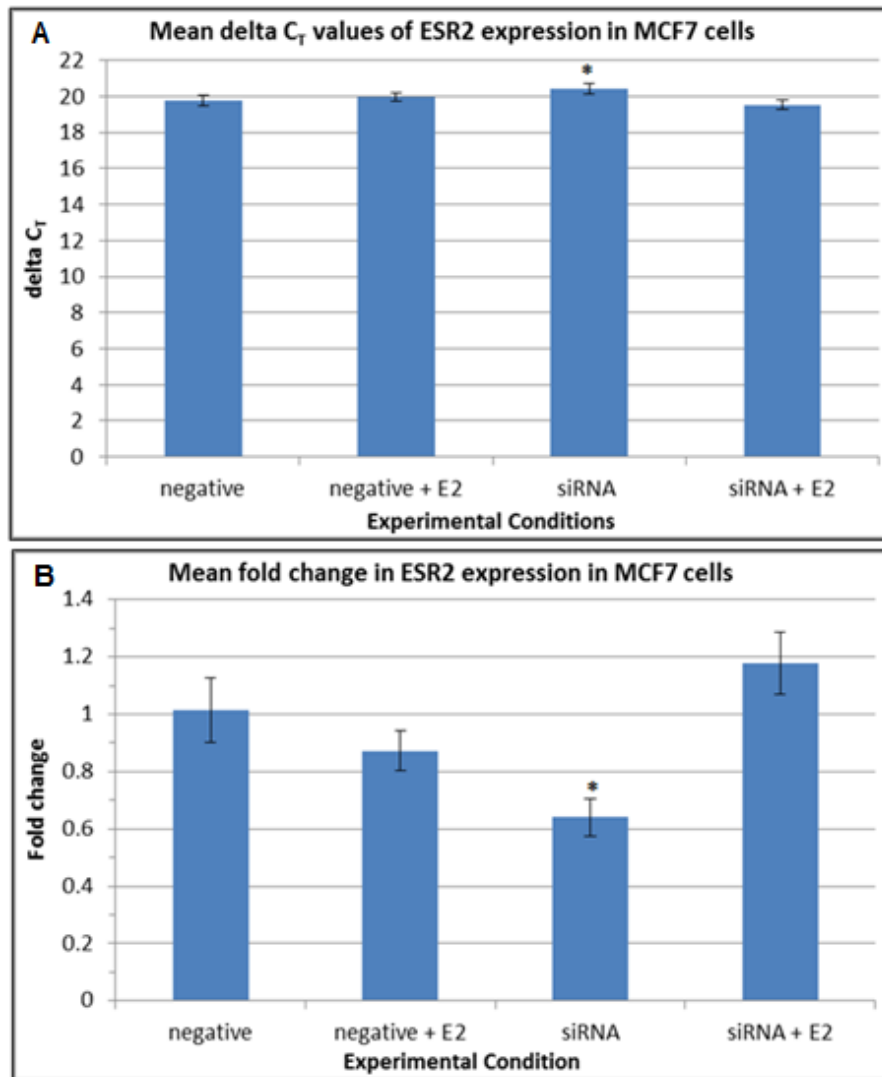


Figure 9 – Levels of oestrogen receptor β (ER β) RNA expression in MCF7 cells transfected with either a negative control siRNA or a combination of two siRNAs targeted against ESR2; and either treated with 10nM oestradiol or not, in terms of delta C_T values (A) and fold change (B). Data were obtained from q-PCR and are mean values (n = 3 for the negative without oestradiol and negative with oestradiol experimental groups; n = 4 for the siRNA without oestradiol and siRNA with oestradiol groups), with error bars showing standard deviation (A) and standard error of the mean (B). * = $p < 0.05$ (Student's t-test)

3.2.1.2 Ret

The MCF7 cells from the above experimental conditions were also assayed for RNA expression of Ret, in order to see if Ret RNA expression was significantly affected by ER β expression and/or the presence of oestrogen in this cell line. However, the Ret RNA expression levels were so low that they could not be detected in the majority of samples. Consequently, these data could not be presented.

3.2.1.3 Cyclin D1

The MCF7 cells from the above experimental conditions were also assayed for RNA expression of cyclin D1, in order to see if levels of ER β RNA and/or the presence of oestradiol had any significant effect on the levels of cyclin D1 RNA in this cell line.

Cyclin D1 was investigated because it is a known oestrogen-responsive gene, and is critical for progression through the cell cycle [2, 6, 18, 21, 25-27]. Fig. 10 below shows the level of cyclin D1 RNA expression in MCF7 in the experimental conditions as above: negative, negative + E2, siRNA, and siRNA + E2. Fig. 10A shows the mean delta C_T values of cyclin D1 RNA expression in each experimental group, with error bars representing SD. The mean delta C_T value for the negative group was 11.3 ± 0.5 , which was statistically unchanged in all experimental groups. However, the mean delta C_T values trended towards an increase to 11.9 ± 0.4 and 11.9 ± 0.3 in the siRNA and siRNA + E2 groups, respectively, which was approaching significance ($p = 0.12$ and 0.09 , respectively). Fig. 10B shows the mean fold change in cyclin D1 RNA expression, with error bars representing SEM. The mean fold change in the negative group was 1.0 ± 0.2 , which was statistically unchanged in all experimental conditions. However, in the siRNA and siRNA + E2 groups, the mean fold change trended towards a decrease to 0.7 ± 0.1 and 0.7 ± 0.1 , respectively, which was approaching significance ($p = 0.14$ and 0.11 , respectively).

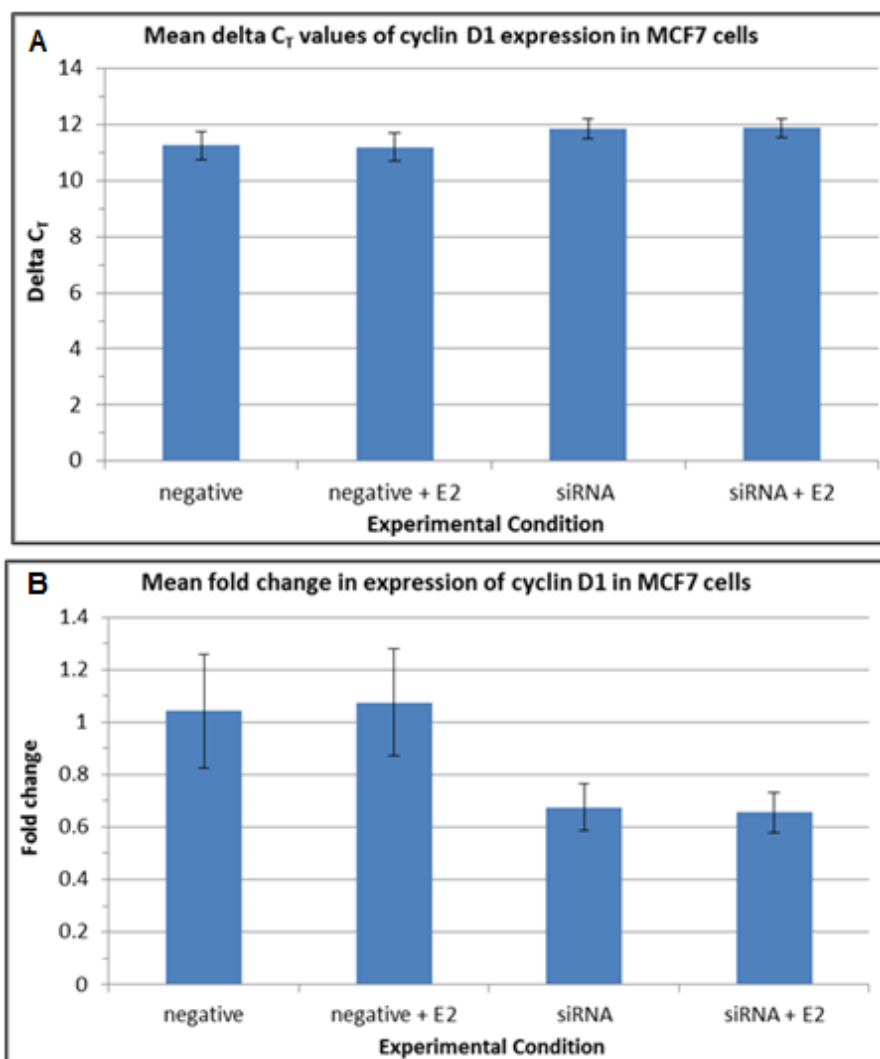


Figure 10 – Levels of cyclin D1 RNA expression in MCF7 cells transfected with either a negative control siRNA or a combination of two siRNAs targeted against ESR2; and either treated with 10nM oestradiol or not, in terms of delta C_T values (A) and fold change (B). Data were obtained from q-PCR and are mean values ($n = 3$ for the negative without oestradiol and negative with oestradiol experimental groups; $n = 4$ for the siRNA without oestradiol and siRNA with oestradiol groups), with error bars showing standard deviation (A) and standard error of the mean (B).

3.2.1.4 IGF-1R

The MCF7 cells from the above experimental conditions were also assayed for RNA expression of IGF-1R (insulin-like growth factor type 1 receptor), in order to see if the levels of IGF-1R RNA are significantly affected by levels of ER β RNA and/or the presence of oestradiol in this cell line. IGF-1R was investigated because it is known to be an oestrogen-responsive growth factor receptor and is involved in cell growth

and proliferation [2, 6, 10, 12, 18, 19, 21]. Fig. 11 below shows the level of insulin-like growth factor type 1 receptor (IGF-1R) RNA expression in MCF7 cells in the experimental conditions as above: negative, negative + E2, siRNA, and siRNA + E2. Fig. 11A shows the mean delta C_T values of IGF-1R RNA expression in each experimental group, with error bars representing SD. In the negative group, the mean delta C_T value was 11.6 ± 0.6 , which was statistically unchanged in each experimental condition. However, all three experimental conditions did trend towards a decrease in IGF-1R RNA levels, which was trending towards significance ($p = 0.64, 0.69$, and 0.43 , for negative+E2, siRNA, and siRNA+E2, respectively). Fig. 11B shows the mean fold change in IGF-1R RNA expression, with error bars representing SEM. The mean fold change in the negative group was 1.1 ± 0.3 , which was statistically unchanged in all experimental conditions. However, the siRNA + E2 group trended towards an increase in IGF-1R levels, which trended towards significance ($p = 0.48$).

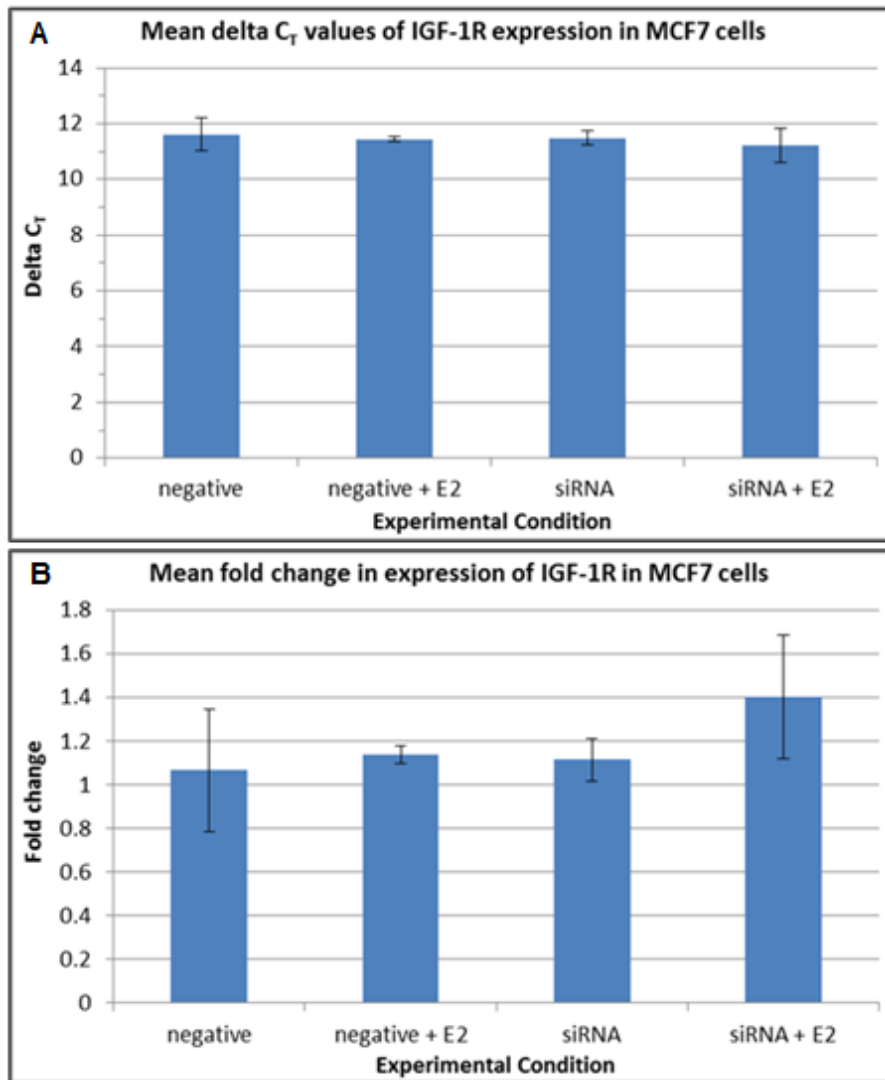


Figure 11 – Levels of insulin-like growth factor type 1 receptor (IGF-1R) RNA expression in MCF7 cells transfected with either a negative control siRNA or a combination of two siRNAs targeted against ESR2; and either treated with 10nM oestradiol or not, in terms of delta C_T values (A) and fold change (B). Data were obtained from q-PCR and are mean values ($n = 3$ for the negative without oestradiol and negative with oestradiol experimental groups; $n = 4$ for the siRNA without oestradiol and siRNA with oestradiol groups), with error bars showing standard deviation (A) and standard error of the mean (B).

3.2.2 Western blot

MCF7 cells were transfected with a negative control siRNA or a combination of two different anti-ESR2 siRNAs (s4826 and s4827), and then treated either with or without 10nM oestradiol, in order to see if significant knockdown of ER β could be achieved at the protein level in this cell line, and whether the presence of oestrogen

has any effect on the amount of ER β protein expressed. Fig. 12 below shows 50 μ g protein in each well. However, there was not enough protein harvested from the cells transfected with negative control siRNA and treated without oestradiol, so it could not be loaded into the gel. The membrane was blocked in BSA (see Figure 12C and Section 2.9) and exposed to X-ray film for 1 minute, as background staining was high with prolonged exposure. ER β was detected around 75kDa, as there was no consistent band detected closer to 55kDa, even at a longer exposure, while β -actin was still detected around 50kDa, and bands were analysed using densitometry (see Fig. 12C and Section 2.9.1). Fig. 12A shows the level of ER β protein expression in MCF7 cells transfected with either negative control siRNA or a combination of two different siRNAs targeted against ER β (s4826 and s4827), and either treated with or without 10nM oestradiol. Densitometry analysis (Fig. 12C) showed that there was no significant difference in ER β expression in the three experimental groups, although the two groups transfected with anti-ESR2 siRNA trended towards weaker bands, which were approaching significance ($p = 0.20$ and 0.12 for the siRNA and siRNA + E2 groups, respectively). However, there was no statistical difference between ER β expression in the samples transfected with siRNA and then treated with or without oestradiol. Fig. 12B shows fairly consistent β -actin bands, indicating even protein loading.

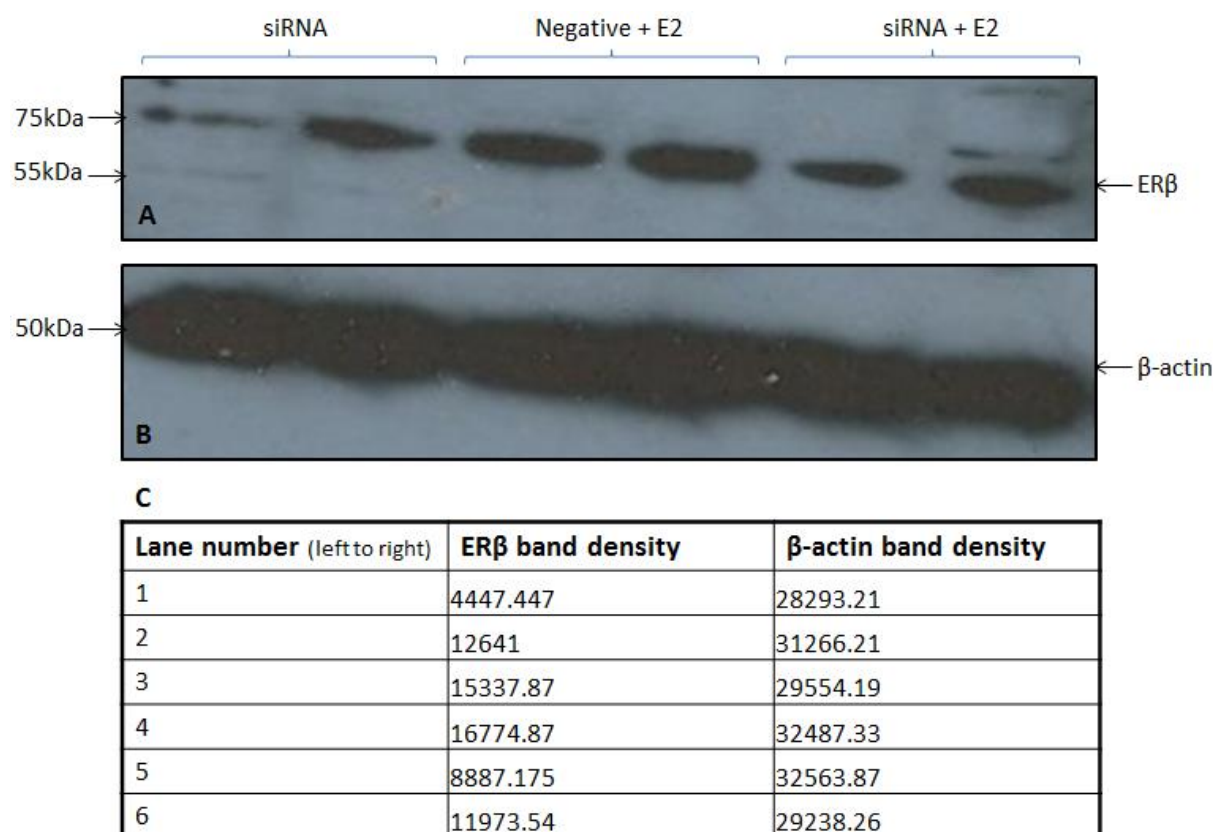


Figure 12 – Western blot showing oestrogen receptor β (ER β) protein expression (A) and β -actin protein expression (B) in MCF7 cells transfected with either a negative control siRNA or a combination of two siRNAs targeted against ESR2; and either treated with or without 10nM oestradiol. C shows the densitometry values of the ER β and β -actin bands in each of the lanes, numbered 1-6 from left to right.

4.0 Discussion

4.1 Success of ER β knockdown

In order to establish potential effects of inactive ER β mutations on tumourigenesis, we needed to experimentally replicate the loss of functional ER β . This was done by transfecting SW1736 (a human thyroid carcinoma cell line) and MCF7 (a human breast carcinoma cell line) cells with anti-ESR2 siRNA and examining levels of ER β RNA and protein expression via q-PCR and Western blotting, respectively.

While the data in Figs. 5 and 8 showed a trend towards decreased ER β expression with siRNA transfection at the RNA and protein level, respectively, in SW1736 cells,

only Fig. 7 showed a significant reduction in ER β protein expression in these cells when transfected with siRNA 1 (s4826) or siRNA 2 (4827) ($p < 0.05$). The fact that a significant reduction in ER β protein expression was only seen in Fig. 7 and not Fig. 8, even though Fig. 8 was loaded with double the protein concentration of all samples from Fig. 7, was unexpected. However, there was a substantial time period between running the two blots, with the samples being stored at -20°C, so it is possible that there was some protein degradation during that time, especially during the thawing process.

Conversely, Fig. 12 only shows an insignificant trend towards decreased ER β protein expression in the MCF7 cell line. Fig. 9 shows that there was significant knockdown of ER β RNA expression in this cell line when transfected with a combination of two anti-ESR2 siRNAs (s4826 and s4827) and then treated without oestrogen.

These data show that there was some knockdown of ER β in both cell lines at the RNA and protein level. However, this was only significant in two experiments.

Consequently, the knockdown conditions needs to be optimised to consistently achieve knockdown at both the RNA and protein level in both cell lines, in order for subsequent experiments to be validated. However, time constraints prevented this from occurring.

4.2 Effect of ER β knockdown on the expression of Ret, cyclin D1 and IGF-1R

The effect of reduced ER β expression on the RNA levels of the proto-oncogene Ret, the cell-cycle regulator cyclin D1, and the growth factor receptor IGF-1R, was then investigated via quantitative polymerase chain reaction (q-PCR).

Fig. 6 shows that there was no consistent, significant change in Ret RNA expression when ER β levels were reduced (albeit insignificantly) in the SW1736 cell line.

Furthermore, the lack of data for Ret RNA expression in the MCF7 samples shows that Ret expression was very low in all of these samples, regardless of the amount of ER β RNA expression (Fig. 9).

Furthermore, Fig. 11 does not show a significant change in IGF-1R RNA expression in MCF7 cells, despite a significant reduction in ER β RNA (Fig. 9).

Fig. 10 shows a trend towards a decrease in expression of cyclin D1 RNA when ER β RNA levels were significantly reduced in MCF7 cells (Fig. 9). This trend was approaching significance ($p = 0.13$ on average).

Together, these data show that significant ER β RNA knockdown did not have any significant effect on the expression of Ret, cyclin D1, or IGF-1R, at the RNA level in MCF7 cells, or the expression of Ret RNA in SW1736 cells. This suggests that the expression of these genes are unaffected by the level of ER β expression in this cell line, although the trend towards a decrease in cyclin D1 in MCF7 cells was approaching significance, suggesting that cyclin D1 expression may depend on ER β expression. Further repeats would be needed to confirm this, but time constraints prevented this from occurring.

It has been shown previously that oestrogen induces Ret expression and drives cell proliferation [10]. However, these data suggest that a reduction in functional ER β expression may not be solely responsible for alterations in Ret expression in the initiation and/or progression of MTC, as there is no consistent trend between ER β and Ret expression observed. Furthermore, from this data, it seems unlikely that MTC develops through a pro-proliferative change in cyclin D1 expression with

reduced functional ER β expression. Overexpression of cyclin D1 is often observed in cancers, as this drives transition through the cell cycle [6, 21, 25-27], and in order to support the idea that ER β keeps cell proliferation in check, we should see an increase in cyclin D1 expression when ER β is knocked down [2, 18], instead of the decrease observed in this data. These data show that only IGF-1R trended towards an increased RNA expression when ER β was knocked down in the MCF7 cell line. This was to be expected, as it has been found previously that IGF-1R expression is associated with increased cell proliferation and tumour progression [12], and that IGF-1R is involved in ligand-independent ER α signalling [19], which will go unregulated due to ER β knockdown [2, 6, 10, 18, 21]. Consequently, it may be that this is the pathway in which MTC is initiated or progresses when ER β is mutated. However, as this change was not significant, this cannot be confirmed, so further repeats are needed, as time constraints prevented this from happening.

4.3 Effect of oestrogen on ER β , Ret, cyclin D1 and IGF-1R expression

Fig. 9 suggests that oestradiol reduces ER β RNA expression in negative siRNA-transfected MCF7 cells; while it increases ER β RNA expression in anti-ESR2 siRNA-transfected cells, although this was insignificant. Fig. 12 does not show any effect of oestradiol on the expression of ER β protein in MCF7 cells transfected with siRNA. Neither of these figures shows a significant effect of oestrogen on ER β expression at the RNA or protein level, suggesting that oestrogen does not regulate ER β expression. Alternatively, it may be that because of the knockdown level of ER β in both cell lines, there is less ER β to be upregulated by the presence of oestradiol.

As there were negligible amounts of Ret RNA expression across MCF7 cells from all experimental conditions, no data could be presented. This suggests that oestrogen has no effect on Ret RNA expression.

Fig. 10 shows that oestradiol does not affect the level of expression of cyclin D1 RNA in MCF7 cells, as there is little difference between the delta C_T values and fold change levels between the respective groups.

Fig. 11 shows that oestradiol does not have an effect on IGF-1R RNA expression in MCF7 cells with normal ER β RNA expression, but that oestradiol seems to have a greater effect on increasing IGF-1R RNA expression in MCF7 cells with a significantly reduced ER β RNA expression (Fig. 11).

Together, these data suggest that oestrogen has no significant effect on the amount of ER β , Ret, cyclin D1, or IGF-1R RNA expression. However, it has been found previously that oestrogen acting through ER α has a pro-proliferative role, whereas it has an anti-proliferative role when acting through ER β , through reducing the transcriptional activity of ER α [6, 18, 20]. This has been shown previously, as activation of ER β signalling through the use of its agonist diarylpropionitrile (DPN) and adenovirus-mediated transfection resulted in reduced cell proliferation and migration, due to reduced expression of cyclin D1 [2] and the anti-apoptotic protein Bcl-2 [6, 21], and increased apoptosis, due to increased expression of the pro-apoptotic protein caspase-3 [2]. Consequently, when ER β is knocked down, there should be increased expression of the various genes activated by ER α signalling which are responsible for cell proliferation, growth and migration, such as Bcl-2, which has been shown previously [6, 21]. However, as there were trends towards decreased RNA expression of these oestrogen-responsive genes, it may be that

there was some RNA degradation and/or degradation of the fluorescence signal from the Taqman assays occurring, as technological problems meant that the plates spent longer in the light than normal (Taqman assays are slightly light sensitive), and had to be stored at -20°C, so there may have been some RNA degradation during the thawing process. Consequently, these experiments should be repeated, but time constraints prevented this from occurring.

4.4 Areas for future work

While there were replicates in each experimental condition in each experiment carried out ($n = 4$ for RNA transfection; $n = 2$ for protein transfection) and duplicates of each sample were assayed (see Section 2.0), and the mean of these was taken, data were only collected once from each experiment, as time and resource constraints only allowed one repeat to be carried out. In order to improve the validity of these results, data needs to be collected from further repeats of each experiment. Furthermore, the effect of 10nM oestradiol on ER β , Ret, cyclin D1 and IGF-1R RNA expression and ER β protein expression in negative control or anti-ESR2 siRNA-transfected SW1736 cells should also be investigated using q-PCR and Western blotting, respectively, as time and resource constraints prevented this from occurring. It would also be beneficial to investigate the effect of anti-ESR2 siRNA and oestrogen on the level of Ret, cyclin D1 and IGF-1R protein expression by Western blotting in both cell lines, as time constraints did not allow this to happen. It would also be worth investigating the consequent expression of other genes known to be oestrogen-responsive and/or be involved in regulation of the cell cycle or migration, such as the growth factor receptors VEGFR and EGFR [3-5, 12, 14], the transcription factor NF κ B [28], the anti-apoptotic protein Bcl-2 [6, 21], the proto-

oncogene c-myc [2], or the cytoskeletal-linker proteins ezrin and moesin [29, 30], in order to understand this pathway better, as time and resource constraints did not facilitate this.

To further investigate the role of ER β and oestrogen in tumourigenesis, the level of cell proliferation and migration in both cell lines with either wild-type or knockdown levels of ER β , and treated with or without oestrogen, should be examined, as time constraints prevented this from happening. This could be done by MTS or MTT assays, or BrdU assays to see if reduced ER β expression affects cell proliferation and migration, and whether the presence of oestrogen has any impact. If ER β knockdown significantly increases the proliferation and migration of these cell lines, it would show that mutated, dysfunctional and/or reduced levels of ER β can be responsible for the initiation and progression of MTC.

In order to confirm that ER β is essential for cell cycle regulation and that a loss of functional ER β can be responsible for developing MTC, these cell lines should be transfected with wild-type ER β to examine the effects of over-expressing ER β .

These cells should have significantly reduced proliferation and migration compared to control in order to support this hypothesis. Furthermore, transfection with mutated forms of ER β should result in increased rates of cell proliferation and migration.

5.0 References

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